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ABSTRACT

Influence of Herpes Simplex Virus Latency Associated Transcript (LAT) on the Distribution of Latently Infected Neurons

Andrea S. Bertke, Doctor of Philosophy, 2007

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Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are similar viruses with several notable differences. While both viruses establish latency in sensory ganglia and reactivate to cause recurrent disease, HSV-1 reactivates more efficiently from trigeminal ganglia to cause cold sores or keratitis and HSV-2 reactivates more efficiently from lumbosacral dorsal root ganglia (DRG) to cause genital herpes. Both viruses are capable of causing central nervous system (CNS) disease, but HSV-1 CNS infections typically manifest as severe necrotizing encephalitis while HSV-2 is more commonly associated with relatively benign meningitis. Potential mechanisms for type-specific differences between HSV-1 and HSV-2 have not been closely examined, although the latency-associated transcript (LAT) of HSV plays a critical role in the establishment of latency and site-specific reactivation and may have regulatory influence over viral replication and spread. In the work presented here, HSV-1 and HSV-2 were evaluated using the guinea pig model of infection to characterize differences in viral spread, replication, and the establishment of latency in the peripheral and central nervous systems. Chimeric viruses and a LAT promoter-deleted virus were also evaluated to determine the role of LAT in

type-specific differences between HSV-1 and HSV-2, and also to determine the specific region of LAT that contributes the critical elements for HSV-2 reactivation. The major findings of this thesis are: 1) HSV-1 and HSV-2 spread to different regions of the central nervous system, preferentially utilizing different autonomic pathways in addition to sensory pathways; 2) type-specific LAT regions influence viral replication and gene expression differently in the DRG and the spinal cord mediated by host factors in specific types of neurons, contributing to differences in viral spread, neurotropism, severity of disease, and latent viral load; and 3) LAT exon 1 contributes the critical elements for type-specific reactivation of HSV-2. Taken together, the results provide insight into potential mechanisms for type-specific differences between HSV-1 and HSV-2, providing a basis for additional molecular analyses of the regulatory control of type-specific latency and reactivation as well as CNS involvement after peripheral HSV infections. In addition, the results suggest modifications to the current model of HSV pathogenesis.

Influence of Herpes Simplex Virus Latency-Associated Transcript (LAT) on the Distribution of Latently Infected Neurons

by

Andrea S. Bertke

Dissertation submitted to the Faculty of the

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Chapter 1

Introduction

Herpes Simplex Virus - Clinical and Epidemiological Background

Infections with herpes simplex virus (HSV) are widespread. Herpes simplex virus type 1 (HSV-1), a ubiquitous pathogen infecting approximately 90% of the United States population, is typically associated with orofacial lesions but is also the most common infectious cause of blindness in developed countries, the most common cause of fatal sporadic encephalitis, and has been implicated in other neurological disorders such as Bell's Palsy and vestibular neuritis (46). Genital herpes infection is typically caused by herpes simplex virus type 2 (HSV-2), but HSV-1 is now estimated to be the cause of 30% of new genital infections due to changes in sexual behavior, particularly among younger populations. HSV-2 infections of the central nervous system (CNS) typically manifest as recurrent meningitis, subacute encephalitis, myelitis, and forms resembling psychiatric syndromes (16). HSV-2 also causes disseminated disease in neonates and immunocompromised individuals. In the United States, the prevalence of HSV-2 has increased more than 30% since the late 1970's, currently infecting approximately 25% of the population (19). Epidemiological surveys demonstrate that most infected individuals are unaware of their positive status because both primary and recurrent disease can be atypical or subclinical. Ignorance of infection and asymptomatic viral shedding have contributed to the increasing transmission of the virus.

Herpes simplex virus is a large enveloped DNA virus of the family Herpesviridae. The virus enters and replicates in the epithelium where it gains access to free sensory

nerve endings (Figure 1). HSV is transported by retrograde axonal transport to sensory neuronal cell bodies within the ganglia innervating the site of inoculation, where the viral genome circularizes into an episomal molecule and establishes life-long latency. The sensory neurons in which the virus establishes latency have a single, bifurcated axon with one branch extending into the periphery and the second branch into the spinal cord. During either primary or recurrent disease, the virus can transport along the spinal cord branch of the axon to reach the central nervous system. Viral latency has been described in central nervous system neurons, although reactivation is not thought to occur from these CNS neurons.

Episodes of reactivation in response to various stress stimuli may cause recurrent peripheral disease at or near the original site of inoculation. HSV-1 reactivates more efficiently from trigeminal ganglia innervating the face and causes most cases of recurrent ocular or orofacial HSV infection. Most cases of recurrent genital herpes are caused by HSV-2, which preferentially reactivates from sacral dorsal root ganglia (53). The precise mechanisms governing the establishment and maintenance of latency and the molecular factors involved in reactivation have yet to be fully explained. The majority of studies exploring HSV latency and reactivation have utilized HSV-1 as a model system, with the assumption that findings with HSV-1 will also apply to HSV-2. However, notable clinical differences such as preferred anatomical sites of recurrence and differences in central nervous system manifestations demonstrate that aspects of HSV pathogenesis differ considerably between HSV-1 and HSV-2.

The clinical presentations of HSV central nervous system infections suggest that neuronal spread of the virus may be differentially regulated between HSV-1 and HSV-2.

HSV-2 is more neurovirulent than HSV-1 but rarely reaches the brain. HSV-1 demonstrates a greater capacity for transneuronal spread to the cerebral cortex. Understanding the differences in the ways HSV-1 and HSV-2 spread through the nervous system is important for effective clinical treatment of peripheral and central nervous system infections caused by HSV. Molecular analysis of the regulatory control of viral spread in the nervous system will also provide insight into methods for the prevention of central nervous system involvement following peripheral HSV infections. In addition, a more complete understanding of the mechanisms underlying the establishment of latency and reactivation of the virus will eventually lead to the prevention of transmission of HSV through the development of antivirals that target the mechanisms of latency establishment and the reactivation process.

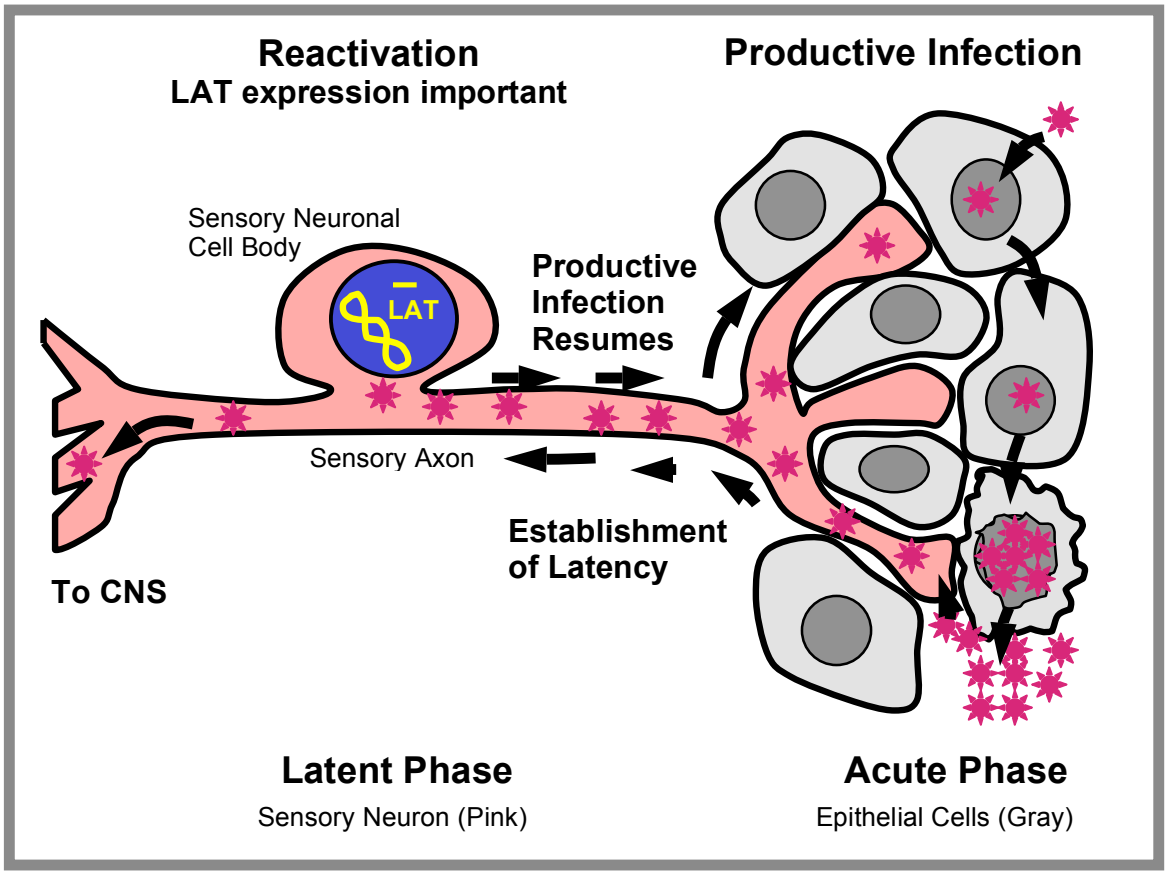


Figure 1. Current Model of HSV Infection and Latency.

Virus replicates in the epithelium (gray cells) where it gains entry into free nerve endings of sensory neurons (pink). The virus is transported by retrograde axonal flow (arrows to the left) to the sensory neuronal cell bodies which lie in the innervating ganglia, trigeminal ganglia (TG) for HSV-1 and lumbosacral dorsal root ganglia (DRG) for HSV-2, although HSV-1 is capable of establishing latency in DRG as well. In the neuronal cell bodies in the ganglia, the virus establishes life-long latency and expresses the latency-associated transcript (LAT). In response to various stimuli, such as stress, fever, or UV light, the virus may periodically reactivate from the sensory neurons in the DRG and is transported by anterograde axonal flow (arrows to the right) back to the peripheral epithelium to cause recurrent disease at or near the original site of inoculation. The specific mechanisms for reactivation, and particularly type-specific reactivation, are not understood but the latency-associated transcript plays a distinctive role. During primary or recurrent disease, the virus is also able to transport via the central axonal branch of the sensory neurons (left of the sensory neuronal cell body) to reach the central nervous system where it can cause CNS disease, typically encephalitis for HSV-1 and meningitis for HSV-2.

Latency-Associated Transcript (LAT)

The HSV genome is a linear strand of double-stranded DNA that consists of unique long and short regions flanked by terminal and internal repeat regions (see Figure 2). The viral genome circularizes upon establishment of latency in the nuclei of sensory neurons, joining the terminal repeats to form a persistent episome. The hallmark of latent HSV infection is the transcription of a single transcription unit termed the latency-associated transcript (LAT), which is transcribed from the repeat regions. Thus, there are two copies of the LAT region in the genome. The LATs were first described in 1984 when Stroop and Fraser detected the transcripts by *in situ* hybridization during both acute and latent infection with HSV-1 (94), and the functions of the LATs are still under investigation. Both HSV-1 and HSV-2 genomes transcribe an approximately 8 kb primary transcript which is spliced into stable nuclear RNAs, characterized as 1.9 and 1.5 kb introns for HSV-1 and a single 2.2 kb intron for HSV-2 (31, 48, 116). The LAT primary transcript runs antisense to an immediate early viral transactivator (ICP0) and a neurovirulence factor (ICP34.5). With the exception of the LAT promoters and the regions overlapping ICP0 and ICP34.5, there is little discernible similarity between the HSV-1 and HSV-2 LAT sequences (68). HSV-1 and HSV-2 contain greater than 80% identity within their promoters and the coding regions of ICP0 and ICP34.5. Both viruses also contain similar splice donor and acceptor sequences flanking their respective introns.

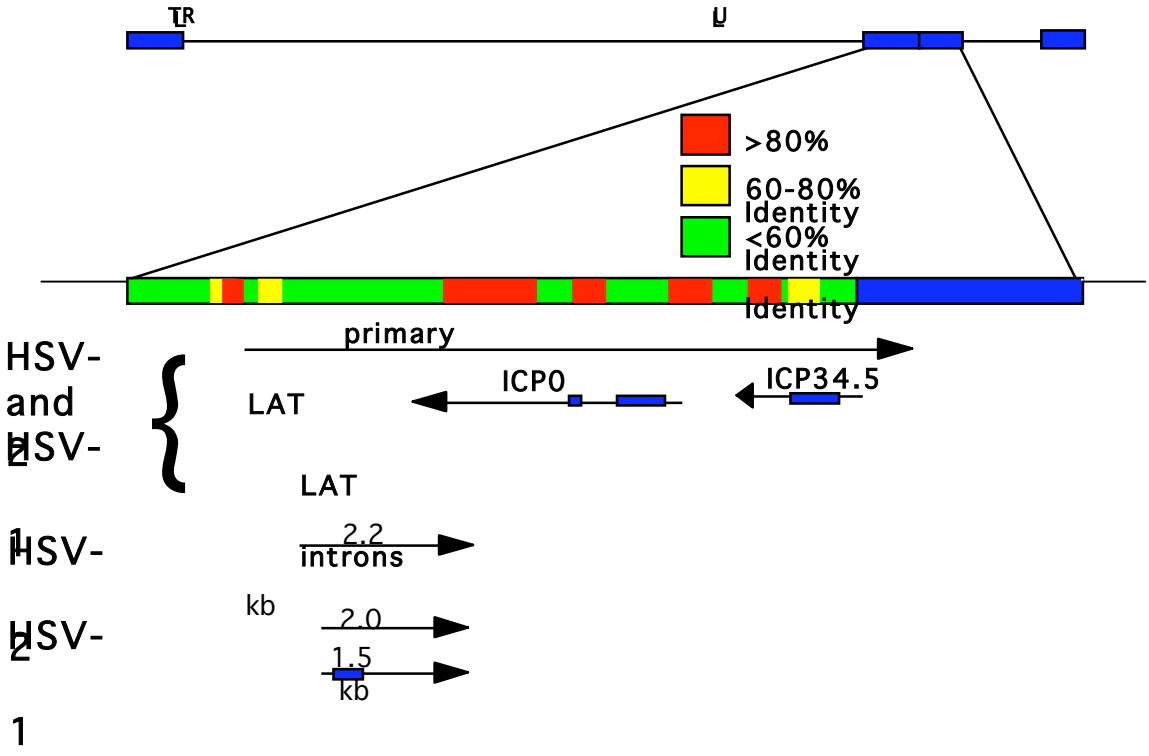


Figure 2. Homology between HSV-1 and HSV-2 LAT regions.

Herpes simplex virus (HSV) is an approximately 152 kb enveloped DNA virus. The genome consists of unique long and short regions (U_L and U_S) flanked by terminal (TR_L/TR_S) and internal repeats (IR_L/IR_S). The virus circularizes upon entry, joining TR_L and TR_S. The latency-associated transcript (LAT) is transcribed from these repeat regions so there are two copies present in the genome. The gene overlaps a viral transactivator, ICP0, and a neurovirulence factor, ICP34.5, on the opposite strand. HSV-1 and HSV-2 both transcribe an ~8 kb primary transcript which is spliced into stable nuclear RNAs referred to as the LAT introns. The primary transcript of HSV-2 is spliced into a single 2.2 kb intron and the HSV-1 primary transcript is differentially spliced into two introns characterized as 1.5 and 2.0 kb long. There is little discernible similarity between the LAT regions of HSV-1 and HSV-2 except in the promoter and in the regions overlapping ICP0 and ICP34.5 coding regions (shown here in red). The blue boxes on the ICP0 and ICP34.5 transcripts and the 1.5 kb HSV-1 LAT intron represent regions that are spliced out of the RNA transcripts.

Mechanism of Action of LAT

LAT plays a role in the reactivation of the virus from latency, although it is unclear whether LAT influences the reactivation process directly or acts indirectly by influencing the establishment of latency. In some models, reactivation frequency correlates with the number of latently-infected neurons (89) and LAT appears to influence the establishment of latency, although there is no apparent correlation between the level of LAT transcript expression and recurrence frequency (12). In both mice and rabbits, LAT-null mutants appear to establish latency in fewer neurons than wild type HSV-1 (22, 63, 87, 91, 97, 98), although the mechanism by which this occurs is unclear.

Although numerous hypotheses have been proposed, the mechanism by which LAT influences reactivation *in vivo* is still not understood. An antisense mechanism was originally proposed since LAT overlaps ICP0, a viral transactivator thought to be involved in reactivation. However, insertion of Poly(A) sequences at several sites within the LAT region of HSV-1, terminating transcription upstream of the overlap region, has no effect on reactivation (10). Transgenic HSV-2 LAT overlapping the ICP0 transcript by 1,673-bp cannot function in *trans*, arguing against an antisense mechanism (106). *Trans* expression of the 2.0 kb HSV-1 LAT intron does not affect ICP0 mRNA expression, stability, accumulation, splicing, or translation (14). Furthermore, an HSV-1 LAT deletion mutant with the first 1.5 kb of LAT, a region that does not overlap ICP0, reinserted in an ectopic location is phenotypically wild type for reactivation (80, 86). The cumulative data strongly argue against an antisense mechanism between LAT and ICP0 for both HSV-1 and HSV-2.

No convincing evidence has demonstrated a protein translated from the LAT transcript *in vivo* (43). The LAT region contains three potential open reading frames (ORFs), and insertion of linker DNA into ORF2 (32) or mutagenesis of the three ATG sites in the region between the promoter and the 5' splice site of HSV-1 do not effect reactivation (10). Furthermore, the LAT function required for efficient reactivation from latency maps to the first 1.5 kb of the HSV-1 LAT transcript, a region that contains no conserved open reading frames (28). A putative protein was described antisense to the 5' end of the HSV-1 LAT transcript and promoter that was thought to potentially be involved in virulence of HSV-1, although this protein has not been isolated in cell culture or animal tissues (82).

Cumulative evidence suggests a *cis*-acting mechanism by which LAT exerts a large part of its influence. The continuous expression of LAT during latency has been thought to provide a potential mechanism for the inhibition of the lytic cycle. Since the gene for the LAT transcript overlaps ICP0 on the opposite strand, continuous expression of LAT could potentially preclude transcription from the opposite strand by steric hindrance, thus preventing expression of the transactivator, ICP0. Alternatively, LAT transcription could maintain an open chromatin structure to facilitate transcription initiation of productive cycle genes when the appropriate stimulus arises. The LAT promoter and 5' exon region are associated with acetylated histone H3, consistent with transcriptionally permissive chromatin, while histones associated with viral DNA polymerase, ICP27, and ICP4 (early and immediate early genes) are not enriched in acetylation (50), consistent with transcriptionally inactive chromatin. While some studies have shown a continuous increase of LAT accumulation (or reporter under the LAT

promoter) during latency (25, 58), others have demonstrated that LAT accumulates rapidly during acute infection and maintains a steady state level after Day 10 (1, 95). Thus, it is currently unclear if LAT is continuously transcribed during latency and the rate of transcription is equivalent to the rate of degradation, or if the stability of the intron simply allows persistence in the nucleus after acute phase production.

The LAT of HSV-1 does have at least one *trans*-acting function. Recent studies have demonstrated that HSV-1 LAT inhibits apoptosis of neurons, which is hypothesized to enhance the establishment of latency (2, 13, 36, 45, 76, 77, 81). However, HSV-2 LAT has not been shown to inhibit apoptosis of neuronal cells. HSV has several anti-apoptotic genes, including ICP27, ICP22, US3, US5, ICP4, and HSV-1 LAT (4). Only LAT is expressed during latency, suggesting that its anti-apoptotic function in HSV-1 would be important during latency or early reactivation. One study reported that the HSV-1 LAT region expresses a miRNA that inhibits apoptosis by modulation of TGF-β signaling *in vitro* (36), but this miRNA is not expressed by HSV-2. An alternative mechanism for apoptosis inhibition was described in which the first 1.5 kb of the HSV-1 LAT transcript was able to inhibit apoptosis by interfering with caspase activation via modulation of the BCL family of proteins (76). By using RNA to regulate host cell apoptosis, HSV-1 could circumvent the need for the expression of viral proteins during latent infection, helping the virus evade immune detection. Although the effect of HSV-1 LAT on apoptosis has been observed by several labs, the prevention of apoptosis could potentially enhance the establishment of latency by increasing the survival of infected neurons but does not explain the process of reactivation, or more specifically type-

specific reactivation. In HSV-2 infection, apoptosis is prevented by a mechanism that appears not to involve LAT (113).

Considerable controversy surrounds the apoptosis theory due to conflicting data from other laboratories. Several investigators have suggested that LAT may enhance neuronal survival through inhibition of productive infection rather than by prevention of apoptosis (33, 61, 88, 97, 99). Using a mouse model, Sawtell and Thompson found increased neuronal death after infection with several HSV-1 LAT deletion mutants, but found few apoptotic neuronal cells (97). The apoptotic cells present were non-neuronal cells thought to be infiltrating lymphocytes. LAT is able to down-regulate viral replication in mice (33) and decrease the transcription of immediate early genes ICP0, ICP4, and ICP27 in transfection assays (61), suggesting that the LAT may function to inhibit viral replication by regulating the viral lytic gene cycle.

Cumulative evidence supports two potential roles for LAT in the establishment of latency: inhibition of apoptosis and/or inhibition of productive infection. While these two hypothetical functions are not mutually exclusive, both mechanisms result in the survival of infected neuronal cells, which would increase the pool of latently infected cells from which the virus could reactivate.

Site-Specific and Neuron-Specific Differences in the Establishment of Latency

While anatomical site-specific preferences for reactivation have been known for decades, molecular studies characterizing the differences in establishment of latency between HSV-1 and HSV-2 were only performed recently. HSV-1 reactivates more efficiently from trigeminal ganglia, giving rise to recurrent orofacial and ocular disease,

while HSV-2 reactivates preferentially from lumbosacral dorsal root ganglia (DRG), giving rise to recurrent genital disease (53). In addition to preferences for specific ganglia, the viruses also demonstrate preferences for specific subtypes of neurons within the ganglia. HSV-1 preferentially establishes latency in neurons that express a surface molecule recognized by monoclonal antibody (mAb) A5 (hereafter referred to as A5+ neurons) (112). HSV-2 preferentially establishes latency in neurons that express a surface molecule recognized by monoclonal antibody KH10 (hereafter referred to as KH10+ neurons) (65). Monoclonal antibodies A5 and KH10 recognize lactoseries carbohydrates on the surface of specific non-overlapping subsets of nociceptive neurons, which are neurons that respond to noxious heat stimuli. Most A5+ neurons express calcitonin gene-related peptide (CGRP) and TrkA, the high-affinity receptor for nerve growth factor (NGF), while KH10+ neurons appear to be a subset of non-peptidergic nociceptors that respond to glial cell-derived neurotrophic factor (GDNF) and are also recognized by the lectin BSL-IB_4 (26, 54, 65). Both subtypes of nociceptive neurons are present in trigeminal and dorsal root ganglia in approximately the same distribution, with 9% of the neurons labeling with mAb A5 and 16% labeling with mAb KH10 (112). Virus is found in the same proportion of neurons during acute infection, indicating that the virus has equal capabilities of infecting either subtype. By latency, however, HSV-1 latently infected neurons are 68% A5+ but only 2% KH10+. HSV-2 latently infected neurons are only 4% A5+ but 42% KH10+ (65). These data suggest that a greater proportion of A5+ neurons acutely infected with HSV-1 progress to latency and a greater proportion of acutely infected KH10+ neurons follow a productive pathway and die, thus reducing the relative establishment of latency in KH10+ neurons. The reverse appears to

be true for HSV-2, with a greater percentage of KH10+ neurons supporting latent infection while the virus proceeds through the productive cycle in A5+ neurons.

Initial characterization of neuronal specificity did not establish the mechanism by which the virus regulates control over the preferential establishment of latency in A5+ or KH10+ neurons, although the LAT region appears to regulate this preference. A chimeric HSV-2 virus expressing HSV-1 LAT (HSV-2/LAT1) reactivates from trigeminal ganglia similar to wild type HSV-1 and has a reduced reactivation frequency from lumbosacral ganglia, demonstrating that LAT confers anatomical site-specificity of reactivation (114). This chimera, HSV-2/LAT1, preferentially establishes latency in A5+ neurons that support HSV-1 latency, suggesting that LAT also influences the subtype of nociceptive neurons in which HSV establishes latency (65). In a reciprocal experiment, substitution of HSV-2 LAT for native HSV-1 LAT impairs the ability of HSV-1 to reactivate from the trigeminal ganglia (38). Taken together, these data suggest that LAT may regulate anatomical site-specificity of reactivation (trigeminal or lumbosacral dorsal root ganglia) by influencing the establishment of latency in specific subtypes of neurons (A5+ or KH10+) from which the virus can most efficiently reactivate.

Antiviral Effects on Establishment of Latency

Because HSV-2 is uniformly lethal in the mouse ocular model, mice are typically treated with acyclovir or γ-globulin when comparing HSV-2 to HSV-1 in this model. Treatment with acyclovir during acute infection shifts the distribution of HSV-1 latently infected neurons from 68% A5+/2% KH10+ to 25% A5+/12% KH10+ (112). Acyclovir is a guanosine derivative that terminates replication of the virus by incorporation of its

triphosphorylated form into the genome of the replicating virus. Treatment with acyclovir has been shown to reduce the induced reactivation frequency in mice (92), suggesting that by interfering with replication during acute infection, acyclovir can also interfere with the normal establishment of latency. Treatment with γ-globulin during acute infection in mice also reduces the induced reactivation frequency of HSV (104). Mechanistically acyclovir acts on viral replication and γ-globulin acts on cell-to-cell viral spread, suggesting that replication and spread of the virus during acute infection influences the reactivation frequency as well as the distribution of latently infected neurons. Because LAT influences the reactivation frequency as well as the distribution of latently infected neurons, it follows that LAT may play a role in the replication efficiency and spread of the virus to specific subtypes of neurons that are permissive for reactivation.

Neuronal Pathways: Interaction between Sensory Neurons and the Central and Autonomic Nervous Systems

The paradigm of HSV latency is the establishment of latency in sensory neurons in the trigeminal or lumbosacral dorsal root ganglia (DRG). The sensory neurons in which HSV establishes latency have their neuronal cell bodies in the innervating ganglia and project a single bifurcated axon with one branch extending into the peripheral tissues and the second branch into the spinal cord (Figure 3). In the spinal cord, the sensory neurons synapse in the dorsal horn on projection neurons, which transmit the sensory information to the brain, and on interneurons that conduct impulses to efferent motor and autonomic neurons. Some of the interneurons also send dendrites across the midline of

the spinal cord to transmit information to neurons on the contralateral side of the spinal cord.

The female genitalia are densely innervated by both sensory and autonomic neurons (Figure 4). Both sympathetic and parasympathetic nerve endings terminate in the cervicovaginal mucosa. Pre-ganglionic sympathetic neurons are located in the sympathetic nuclei in the lumbar spinal cord and extend axons to the paracervical ganglia (also known as the major pelvic ganglia) adjacent to the cervix. Pre-ganglionic parasympathetic neurons are located in the parasympathetic nuclei in the sacral spinal cord, and also extend axons into the paracervical ganglia, where the autonomic axons synapse on post-ganglionic autonomic neurons. The post-ganglionic neurons extend fibers with free nerve endings directly into the cervicovaginal epithelium.

Sensory and autonomic neurons provide a sensory reflex pathway for the pelvic organs and external genitalia, passing through the central nervous system at the lumbar and sacral levels of the spinal cord. Sensory nerve endings also synapse directly on post-ganglionic autonomic neurons in the paracervical ganglia to modulate signaling cascades within the cells, particularly within the parasympathetic neurons. Neurotransmitters released from sensory neurons directly (in the autonomic ganglia) or indirectly (through interneurons in the CNS) enhance or inhibit stimulation of autonomic neurons. Autonomic neurons also release neurotransmitters that modulate stimulation and signaling cascades in sensory neurons (103). The pathways between sensory neurons, interneurons in the spinal cord, and autonomic neurons provide a continuous feedback mechanism in which sensory and autonomic responses impact the behavior of peripheral, autonomic, and central neurons and their levels of stimulation.

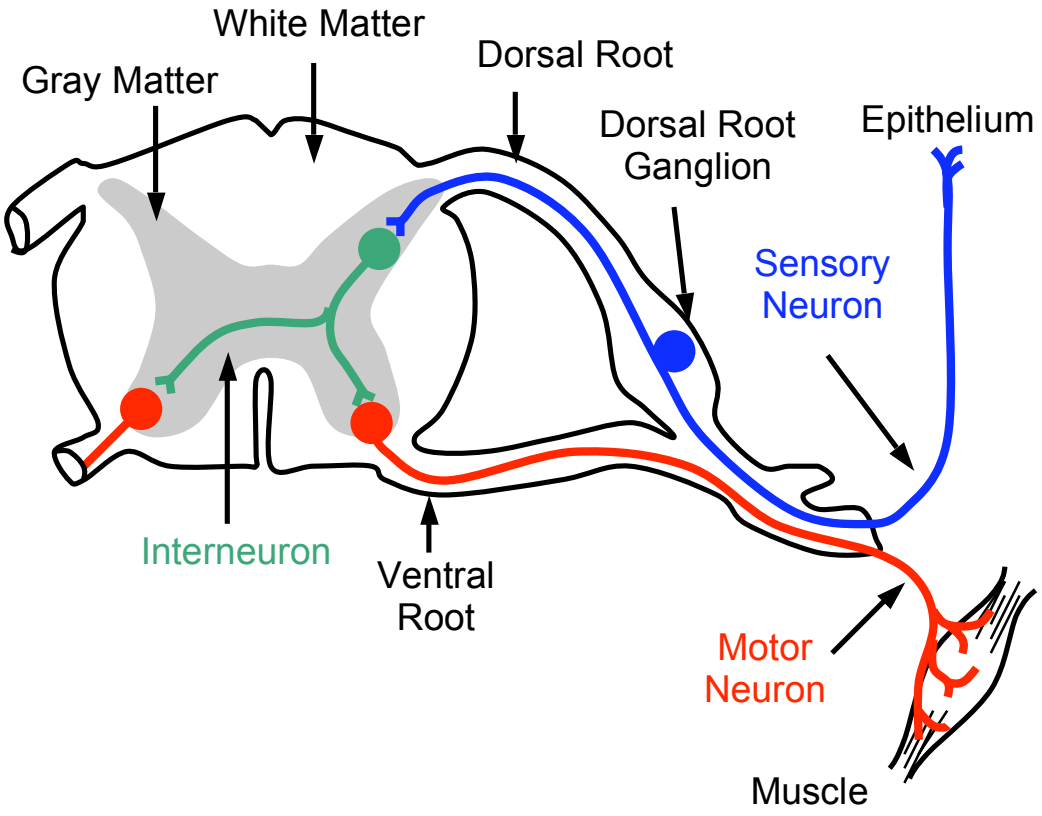


Figure 3. Sensory Reflex Pathway.

The neuronal cell body of a sensory neuron (blue) lies in the dorsal root ganglion, extending one branch of its bifurcated axon into the periphery and the second branch into the dorsal horn of the gray matter in the spinal cord. The axon synapses onto spinal interneurons (green) that relay impulses to motor neurons in the ventral gray matter. Motor neurons (red) exit the spinal cord via the ventral root to conduct impulses to skeletal muscle. Interneurons also relay impulses to autonomic neurons in the spinal cord. *The relationship of the sensory neurons, DRG, and spinal cord are presented here to permit consideration of the neuronal pathways by which the virus spreads.*

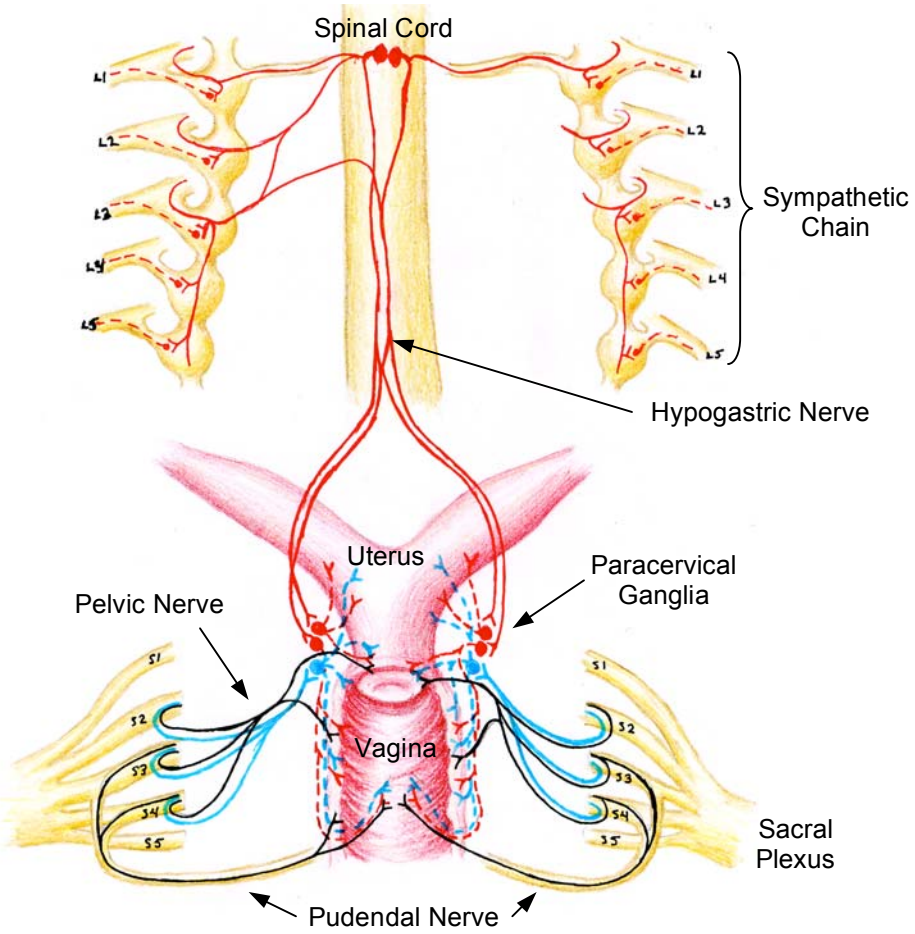


Figure 4. Female Genital Innervation.

The vaginal epithelium is innervated by sensory neurons as well as sympathetic and parasympathetic autonomic neurons. Sensory neurons (black line) terminate in the cervicovaginal epithelium and their axons extend through the pelvic and pudendal nerves to the spinal cord. Pre-ganglionic parasympathetic neurons (solid blue line) originate in the sacral spinal cord and synapse on post-ganglionic parasympathetic neurons (dashed blue line) in the paracervical ganglion. Pre-ganglionic sympathetic neurons (solid red line) originate in the lumbar spinal cord and synapse on post-ganglionic sympathetic neurons (red dashed line) in the paracervical ganglion. Post-ganglionic parasympathetic and sympathetic neurons terminate in the vaginal, cervical, and uterine epithelium. *The relationship of the sensory neurons, spinal cord, and autonomic neurons are described here to permit consideration of alternative neuronal pathways by which the virus may spread.*

Sensory Neurons

The neuronal environment likely plays a significant role in the mechanics of reactivation. Nerve growth factor (NGF) is required to maintain HSV-1 latency in sensory neurons and even short disruptions of NGF result in production of viral proteins and reactivation (39, 110, 111). During latency, 71% of HSV-1 LAT+ neurons express TrkA, the high affinity receptor for nerve growth factor (NGF) (112). The majority of A5+ neurons, in which HSV-1 preferentially establishes latency, are TrkA+ (26, 112). KH10+ neurons, in which HSV-2 preferentially establishes latency, are not responsive to NGF and the effects of NGF have not been examined relative to HSV-2. Downstream second messengers, such as cAMP and protein kinases A and C as well as calcium-dependent kinases, have also been implicated in the reactivation process (93). The cAMP response element binding protein (CREB) and an unidentified nuclear neuronal factor bind the cAMP response element (CRE) in the LAT promoter and reactivation frequency is enhanced by cAMP agonists, suggesting that neuronal factor activation of LAT transcription has a stimulatory effect on reactivation (1, 56). The LAT promoter contains neuronally responsive elements that are bound by as yet unidentified nuclear neuronal factors (5, 58, 105, 107). LAT promoter activity also differs between neuronal cell lines and dorsal root ganglion (DRG) cells, suggesting that DRG cells may have a unique set of transcription factors (24). Additionally, the two subsets of nociceptive neurons in which HSV-1 and HSV-2 preferentially establish latency appear to synapse on second order neurons in distinct regions of the dorsal horn of the spinal cord. A5+ neurons synapse within lamina I in the dorsal horn while KH10+ neurons synapse in lamina II (26, 54). These regions are characterized as having different neuronal pathways that may

influence the ability of the virus to spread within the central nervous system. Cumulative evidence suggests that the neuronal environment may play a critical role in the mechanisms governing establishment of, and/or reactivation from, latency.

LAT Region Critical For Reactivation

Many studies have attempted to determine the specific region of LAT that is responsible for reactivation *in vivo*. HSV-1 and HSV-2 LAT promoter mutants, which express no detectable LAT during latency, demonstrate a universal reduction in the ability to reactivate both spontaneously and after induction in several animal models (7, 9, 17, 23, 41, 49, 56, 66, 78, 80, 90, 100). Several lines of evidence exclude the LAT intron as the critical factor in reactivation. The LAT intron expressed transgenically in mice has no influence on HSV infection or reactivation phenotype (106). Insertion of Poly(A) sequences (10) or λ-phage fragments (8) at several sites within the LAT intron region has no effect on reactivation. Recombinant viruses with deletions within the intron behave like wild type virus (44). Mutation of the splice branch points destabilizes accumulation of the LAT intron but does not influence reactivation (71). Thus, the stable LAT intron is not essential for reactivation.

Recombinant HSV-1 viruses with deletions in the LAT region have mapped the reactivation phenotype to a 1.5 kb portion at the 5' end of the HSV-1 LAT, including the upstream sequence and promoter region (80, 87). Smaller deletions within this 1.5 kb region suggest that the sequence between the promoter and the 5' splice site of the LAT intron may contain essential elements for the reactivation phenotype of HSV-1 (10, 39). A 348- to 371-bp deletion within this region in HSV-1 reduced reactivation after

adrenergic induction in the rabbit ocular model to levels comparable to LAT-negative promoter mutants (10, 22, 39, 59, 85). Various deletions within this region also alter virulence differently in mice and rabbits, suggesting that this region may also influence neurovirulence in a species-specific manner (84), which also implies that specific neuronal factors are involved in the appropriate function of LAT. The region downstream of the LAT transcription start site allows the LAT core promoter to continue functioning during latency and has thus been termed the long-term expression element (LTE) (5, 57). Enhancer functions have been attributed to this region as well (5). Thus, the region that lies between the LAT transcription start site and the 5' splice site of the LAT intron appears to confer regulatory influence over reactivation and possibly neurovirulence in HSV-1. HSV-2 has not been analyzed to determine the specific region critical for reactivation and the potential function of that region.

Nearly all studies on HSV latency have been performed on HSV-1, with the assumption that mechanisms determined in HSV-1 will also hold true for HSV-2. However, HSV-1 and HSV-2 demonstrate critical differences in their pathogenesis. HSV-1 establishes latency preferentially in trigeminal ganglia and reactivates to cause recurrent oral or ocular disease. HSV-2 preferentially establishes latency in lumbosacral DRG and reactivations give rise to genital herpes. HSV-1 is associated more with fatal encephalitis while HSV-2 more commonly causes relatively benign meningitis. Determining differences in the mechanisms by which these two viruses replicate and spread through the nervous system to establish latency and reactivate, as well as cause central nervous system disease, is critical for effective treatment of clinical disease and the development of antivirals and vaccines.

Hypothesis and Specific Aims

Based on available data, LAT has two potential generalized functions for the regulation of reactivation. The LAT region may function to inhibit apoptosis of neuronal cells or prevent progression of the HSV lytic cycle, thus enhancing the survival of infected neuronal cells. The LAT region may also function to regulate the spread of the virus to specific neuronal subtypes that are permissive for the establishment of latency and reactivation. These two general functions are not mutually exclusive. Although several laboratories are exploring the survival functions, the differences in spread between HSV-1 and HSV-2 have not been examined.

Hypothesis

The latency associated transcript (LAT) of Herpes Simplex Virus (HSV) influences the distribution of latently infected neurons by affecting the spread and replication of the virus within the peripheral and central nervous systems. The regulatory element for this influence lies in the region of LAT between the promoter and the 5' splice site of the LAT intron.

Specific Aim #1. Characterize differences between HSV-1 and HSV-2 spread, replication, and establishment of latency in the nervous system. The different clinical presentations of HSV nervous system infections suggest that neuronal spread of the virus may be differentially regulated between HSV-1 and HSV-2. Understanding the different mechanisms by which HSV-1 and HSV-2 cause clinical disease will enhance the ability of physicians to manage HSV-related syndromes. Molecular analysis of viral spread in

the nervous system will also provide insight into methods for the prevention of CNS involvement following peripheral HSV infections.

Specific Aim #2. Determine the influence of LAT region sequences on HSV type-specific differences in spread and the distribution of latently infected neurons. The latency-associated transcript (LAT) of HSV appears to provide regulatory control over the spread of the virus in the nervous system and the establishment of latency in specific subtypes of neurons. Analysis of the regulatory control of viral spread and the establishment of latency in the nervous system will enhance our current knowledge regarding the mechanisms underlying latency establishment and viral reactivation. A greater understanding of these mechanisms will aid in the development of antivirals that specifically target latency establishment, viral reactivation, and transneuronal spread, potentially leading to the reduction of transmission of the virus.

Chapter 2

Materials and Methods

Experimental Approach

The study of herpesvirus latency provides distinct challenges. Although an *in vitro* latency model exists, questions have been raised regarding its reliability and biological relevance. Therefore, animal models must be used to study HSV latency and reactivation. Several animal models have been employed for HSV infection, including mice, rabbits, and guinea pigs. Mice develop acute disease but reactivation must be induced by various stimuli. The mouse model is used primarily for HSV-1 studies because HSV-2 is uniformly lethal unless the mice are given acyclovir, which affects viral replication and the establishment of latency. The rabbit eye provides a latency model with spontaneous reactivation, although the reactivation frequencies are rather low, but rabbits are typically only used for studies with HSV-1 because HSV-2 infection results in high mortality in these animals. The guinea pig develops acute disease with low mortality after infection with either HSV-2 or HSV-1, without the need for acyclovir treatment. In guinea pigs, HSV efficiently establishes latency and reactivates spontaneously with recurrence phenotypes similar to that observed in humans, with HSV-2 reactivating more efficiently than HSV-1 after genital infection. Therefore, the guinea pig provides the most useful model for studying HSV latency and reactivation so was used for the studies presented herein. Unfortunately, all laboratory guinea pig strains are outbred so variability does occur among these animals. With inbred animals that provide data with minimal variability, even small differences between groups can be significant.

However, with our outbred guinea pigs, the variability among these animals demands that any statistically significant differences observed are profound differences rather than minor variances.

The majority of the experiments performed were designed to evaluate aspects of both primary aims of the hypothesis. Instead of addressing each aim separately, experiments were designed to provide information regarding the differences in spread, replication, and the establishment of latency of HSV-1 and HSV-2, as well as provide information regarding the role of different regions of LAT on those differences.

Cells and Viruses

Vero cells were obtained from American Type Culture Collection (ATCC; Rockville, MD), and maintained in minimum essential medium with 10% heat-inactivated FBS and 1% penicillin/streptomycin/L-glutamine (Quality Biologicals, Gaithersburg, MD). HSV-2 strain 333 was obtained from Gary Hayward (Johns Hopkins University, MD). HSV-1 strain 17+ was obtained from Dr. John Hay (SUNY-Buffalo, Buffalo, NY). Virus stocks were produced in Vero cells and plaque-titered in duplicate. To compare one-step growth characteristics of wild type and mutant viruses in cell culture, ~10^6 Vero cells were inoculated in duplicate at time 0 with a multiplicity of approximately 0.1 pfu/cell of each virus. Medium was added after a two-hour adsorption period. At 0, 2, 5, 12, and 20 hours post-inoculation, cells were scraped, freeze-thawed three times, and plaque-titered in duplicate.

Construction of Mutant Viruses

Genome locations for HSV-2 are given relative to the sequence of strain hg52 (27) and for HSV-1 are given relative to the sequence of strain 17+ (69) (refer to Figure 5). To facilitate the generation of mutations in the LAT regions of HSV-1 and HSV-2, an AvrII-AluI fragment of HSV-1 strain 17+ and an SphI-BamHI fragment of HSV-2 strain 333 were each cloned into previously described vectors, Avr-AluΔXho and Sph-Sal-Bam (114). A single base pair change, which did not alter the ICP0 reading frame, was introduced by site-directed mutagenesis, adding an XhoI site to the HSV-1 Avr-Alu clone at position 121259. The LAT regions for all chimeric viruses used in this work are depicted graphically in Figure 37 on page 142.

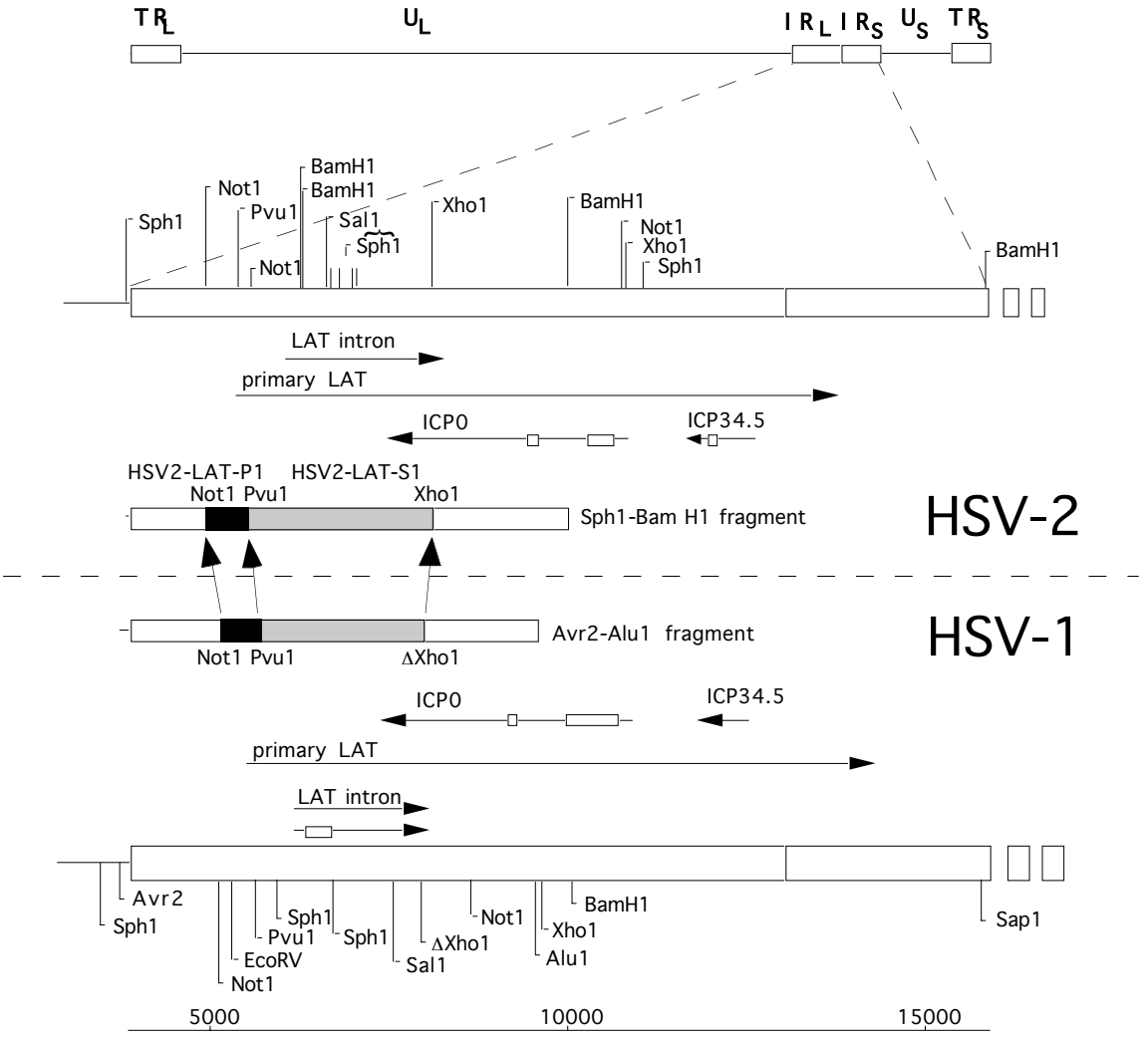
HSV2-LAT-P1 and HSV2-LAT-S1. For chimeric virus HSV2-LAT-P1 (Figure 5), the HSV-1 promoter region from NotI to PvuI (118439-118802) was cloned from the HSV-1 Avr-AluΔXho plasmid into the HSV-2 Sph-Sal-Bam plasmid, replacing the HSV-2 LAT promoter region from NotI to PvuI (119108-119519). For chimeric virus HSV2-LAT-S1, the HSV-1 region from PvuI to the added XhoI site (118802-121259) was cloned into the HSV-2 plasmid, replacing the HSV-2 LAT sequence from PvuI to its native XhoI site (119519-122276). These two plasmids were used to construct chimeric HSV-2 viruses HSV2-LAT-P1 and HSV2-LAT-S1 by homologous recombination after co-transfection of plasmid DNA and parent virus DNA (HSV-2 strain 333) into Vero cells using Lipofectamine reagents (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. After identification of mutant virus, plaque purification was performed until no parent DNA could be detected by Southern hybridization. Three additional blind plaque purifications were performed, purity and correctness of the viruses were verified by

Southern hybridization (Figures 6 and 7), and mutant virus stocks were grown and plaque titered in Vero cells. Sequencing of the mutation junctions further validated the mutant virus sequence. A rescuant was made from HSV2-LAT-S1 using the same method, by co-transfecting mutant viral DNA with the wild type HSV-2 Sph-Sal-Bam plasmid DNA, restoring the native HSV-2 sequences. Amita Patel in our lab constructed HSV2-LAT-P1 and HSV2-LAT-S1 and Andrea Bertke rescued HSV2-LAT-S1, generating HSV2-LAT-S1-R.

The chimeric viruses were characterized for one-step growth in cell culture to verify that an inherent growth defect was not apparent in the viruses that could potentially account for differences in reactivation. In cell culture, ~10^6 Vero cells were infected at time 0 with ~10^5 pfu of each virus. At 2, 5, 12, and 20 hours post-infection, cells were scraped, freeze-thawed three times, and plaque-titered in duplicate. One-step growth kinetics were similar between the chimeric viruses and the wild type viruses (Figure 8).

Figure 5. Construction of HSV2-LAT-P1 and HSV2-LAT-S1.

Genomic and endonuclease restriction sites are shown relevant to HSV-2 strain 333 and HSV-1 strain 17+. The HSV genome consists of uniques long and short (UL and US) regions flanked by internal and terminal repeats (IRL/IRS and TRL/TRS). The primary LAT is transcribed from the repeat regions in the antisense direction from ICP0 and ICP34.5 (transcripts are shown here as lines with arrows indicating the direction of transcription). The primary LAT is spliced into a single stable LAT intron in HSV-2 and two differentially spliced intones in HSV-1. Boxes on the lines denote regions that are spliced out of trancripts and not easily detectable after splicing. For HSV2-LAT-P1, the promoter region from Not1 to Pvu1 in HSV-2 was replaced by the Not1 to Pvu1 promoter region of HSV-1 (shown here in black). For HSV2-LAT-S1, the LAT sequence region from Pvu1 to Xho1 in HSV-2 was replaced by the corresponding LAT sequence region from Pvu1 to ΔXho1 in HSV-1 (shown here as shaded region). The black and shaded regions together represent the region replaced in the previously described HSV-2 333/LAT1 (114) (referred to as HSV-2/LAT1 in this document).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UL
IRL IRS US TRS
Sph1
Not1
BamH1
BamH1
Pvu1
Sal1
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Not1
Xho1
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Not1
Xho1
Sph1
BamH1
LAT intron
primary LAT
ICP0
ICP34.5
HSV2-LAT-P1
HSV2-LAT-S1
Not1 Pvu1
Xho1
Sph1-Bam H1 fragment
HSV-2
Avr2-Alu1 fragment
HSV-1
Not1 Pvu1
ΔXho1
ICP0
ICP34.5
primary LAT
LAT intron
Avr2
Sph1
Pvu1
EcoRV
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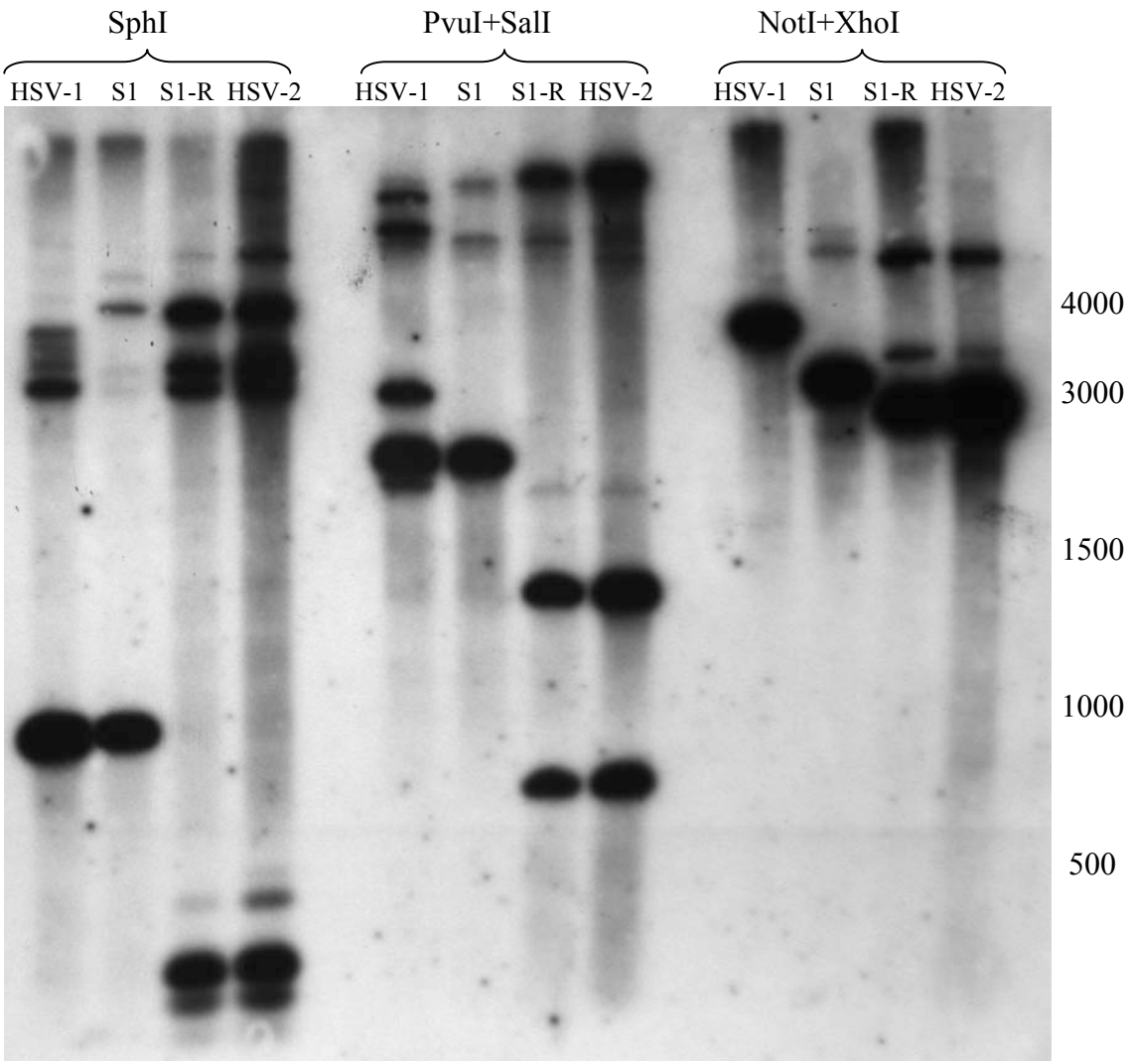


Figure 6. Southern Blot Verification of HSV2-LAT-S1 and HSV2-LAT-S1-R.

The genomes of HSV2-LAT-S1 (S1) and its rescuant HSV2-LAT-S1-R (S1-R) were verified by Southern blot by digesting the viruses with 3 different sets of restriction enzymes and probing the digests with radio-labeled probes specific to the region of the mutation. Each digest gave the expected bands. The last 2 lanes of each group contain the rescuant HSV2-LAT-S1-R and wild type HSV-2 and in each case the bands are the same, indicating a successful rescue.

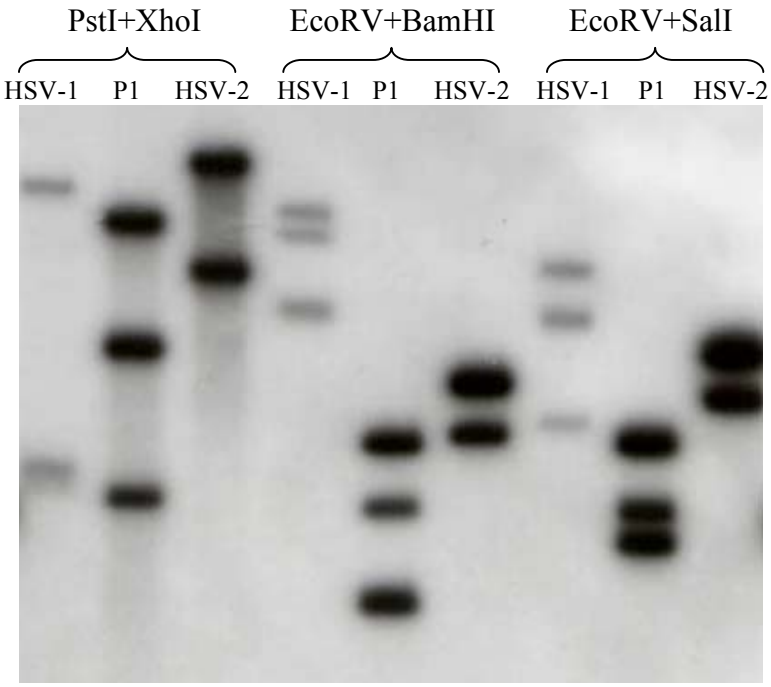


Figure 7. Southern Blot Verification of HSV2-LAT-P1.

The genome of HSV2-LAT-P1 was verified by Southern blot by digesting the chimeric virus HSV2-LAT-P1 (P1) and wild type viruses HSV-2 and HSV-1 with 3 different sets of restriction enzymes and probing the digests with radio-labeled probes specific to the region of the mutation. Each digest gave the expected bands.

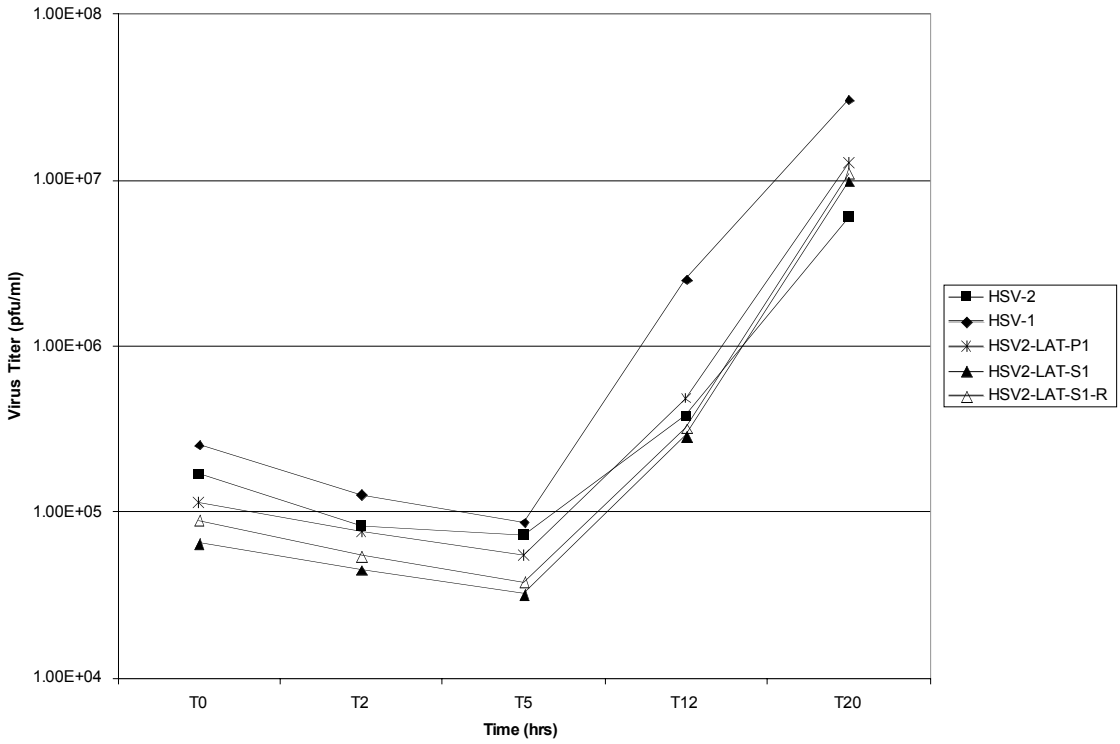


Figure 8. One-Step Growth Curves for HSV2-LAT-P1, HSV2-LAT-S1 and HSV2-LAT-S1R .

To compare one-step growth characteristics of wild type and chimeric viruses, ~10^6 Vero cells were inoculated in duplicate at time 0 with a multiplicity of approximately 0.1 pfu/cell of each virus. Medium was added after a two-hour adsorption period. At 0, 2, 5, 12, and 20 hours post-inoculation, cells were scraped, freeze-thawed three times, and plaque-titered in duplicate. All wild type and chimeric viruses had comparable growth kinetics in cell culture.

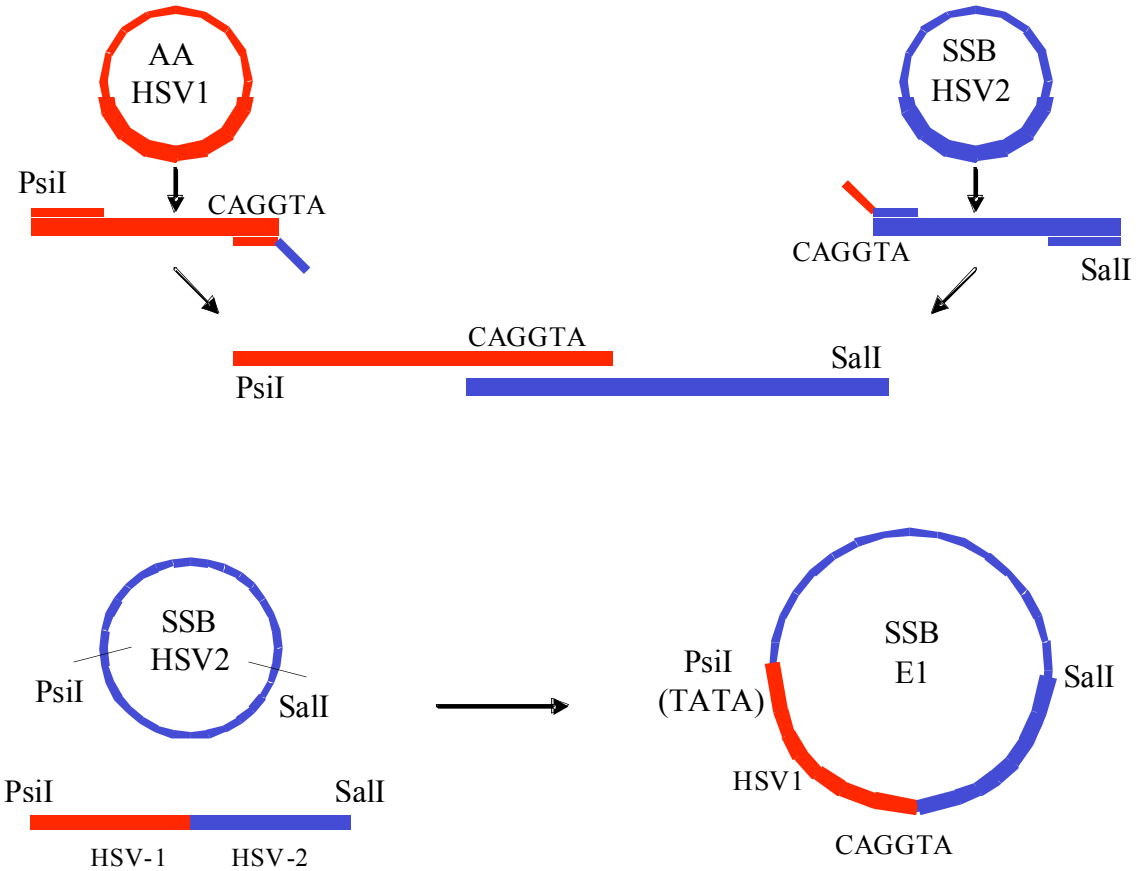
HSV2-LAT-E1. To construct chimeric virus HSV2-LAT-E1 (Figure 9), the region from the TATA box to the LAT intron 5' splice site sequence CAGGTA was amplified by PCR from HSV-1 strain 17+ using primers 5'-GCTGCGTCATCTCAGCCTT - 3' and 5' - CGACCCTAAACC**TACCTG**GAAACGC – 3' and the following cycle conditions: 95°C for 2 min, 35 cycles of 95°C for 20 sec, 50°C for 2 min, and 72°C for 1 min 20 sec, followed by 72°C for 10 min. The CAGGTA sequence is in bold and the underlined segment matches the HSV-2 sequence downstream of the CAGGTA to facilitate overlap PCR in the subsequent amplification. The HSV-2 strain 333 region from the LAT intron 5' splice site sequence CAGGTA to the Sal I restriction site was amplified, using primers 5'–GCGTTTC**CAGGTA**GGTTTAGGGTCG –3' and 5'–ACACAACACGACACGACGC–3' and the following cycle conditions: 95°C for 2 min, 35 cycles of 95°C for 20 sec, 54°C for 2 min, and 72°C for 1 min 20 sec, followed by 72°C for 10 min. The CAGGTA sequence is in bold and the underlined segment matches the HSV-1 sequence upstream of CAGGTA to facilitate overlap PCR. These two PCR products were amplified together by overlap PCR, using primers 5' – GCTGCGTCATCTCAGCCTT - 3' (HSV-1 5'-end primer) and 5' – ACACGACACGACGCGTTTTGC - 3' (HSV-2 3'-end primer) and the following cycle conditions: 95°C for 2 min, 5 cycles of 95°C for 15 sec, 65°C for 15 sec, 72°C for 1 min 20 sec, followed by 30 cycles of 95°C for 15 sec, 54°C for 45 sec, 72°C for 1 min 30 sec, followed by 72°C for 5 min. The resulting product joined the sequences at the splice site sequence CAGGTA, yielding a PCR product consisting of HSV-1 sequences upstream of the CAGGTA and HSV-2 sequences downstream of the CAGGTA. This product was cloned into TOPO vector pCR4, verified by sequencing,

and designated pHSV1-HSV2overlap. pHSV1-HSV2overlap was digested with PsiI and SalI restiction endonucleases to release the overlapped fragment from the TOPO vector. The released fragment was cloned into HSV-2 plasmid SSB, which had also been digested with PsiI and SalI to release the native HSV-2 sequence. The resulting plasmid contained HSV-2 sequences upstream of the TATA box, HSV-1 sequences from the TATA box to the 5' splice site CAGGTA, and HSV-2 sequences downstream of the CAGGTA. This plasmid was designated SSB-E1. Chimeric virus HSV2-LAT-E1 was constructed by homologous recombination after co-transfection of SSB-E1 plasmid DNA and parent virus DNA (HSV-2 strain 333) into Vero cells, as described above. Purity and correctness of the chimeric virus was verified by Southern blot using two sets of restriction endonucleases and a radio-labeled probe specific for the mutation sequence (Figure 10). Sequencing of the complete mutation region further validated the mutant virus sequence. A rescuant was made from HSV2-LAT-E1 using the same method, by co-transfecting mutant viral DNA with the wild type HSV-2 Sph-Sal-Bam plasmid DNA, restoring the native HSV-2 sequences.

The chimeric virus was characterized for one-step growth in cell culture to verify that an inherent growth defect was not apparent that could potentially account for differences in reactivation, as described above. One-step growth kinetics were similar between the chimeric virus and the wild type virus (Figure 11).

Figure 9. Construction of HSV2-LAT-E1.

HSV-1 LAT region from PsiI to the LAT intron 5' splice site sequence CAGGTA was amplified from the AvrII-AluI fragment of HSV-1 in vector (AA HSV1) to isolate PsiI-CAGGTA fragment (red). HSV-2 LAT region from the LAT intron 5' splice site sequence CAGGTA to SalI was amplified from the Sph-Sal-Bam fragment of HSV-2 in vector (SSB HSV2) to isolate the CAGGTA-SalI fragment (blue). HSV-1 PsiI-CAGGTA was joined to HSV-2 CAGGTA-SalI by overlap PCR to generate the HSV-1/HSV-2 PsiI-SalI fragment, which was cloned into the SSB HSV-2 plasmid to replace the native HSV-2 sequences. The new plasmid, SSB-E1, contained HSV-2 sequences up to the TATA (PsiI), HSV-1 sequences from the TATA to the 5' splice site of the LAT intron, and HSV-2 sequences from the 5' splice site (CAGGTA) to the end of the fragment. SSB-E1 was used to generate the chimeric virus HSV2-LAT-E1 by homologous recombination.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PsiI
CAGGTA
SSB
HSV2
CAGGTA
SalI
CAGGTA
PsiI
SalI
SSB
HSV2
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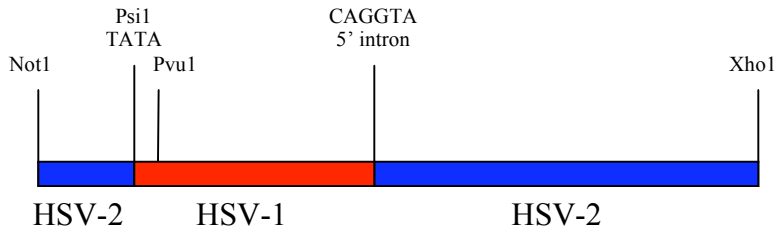
PsiI
SalI
HSV-1
HSV-2
SSB
E1
PsiI
(TATA)
SalI
HSV1
CAGGTA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TATA
CAGGTA
5' intron
Not1
Pvu1
Xho1
HSV-2
HSV-1
HSV-2

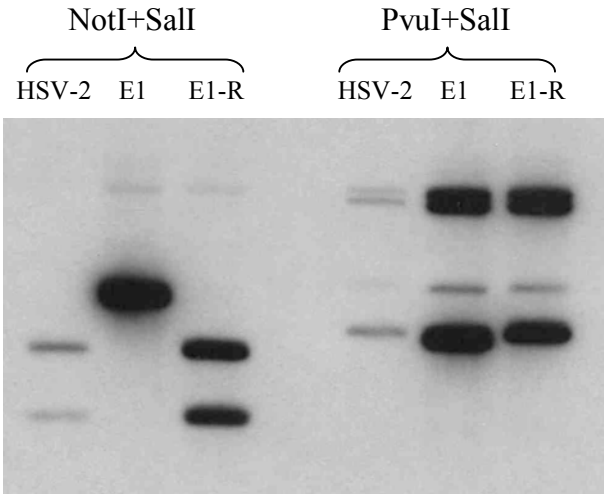


Figure 10. Southern Blot Verification of HSV2-LAT-E1 and HSV2-LAT-E1-R.

The genomes of HSV2-LAT-E1 and HSV2-LAT-E1-R were verified by Southern blot by digesting the chimeric virus (E1), the rescuant HSV2-LAT-E1-R (E1-R), and wild type virus HSV-2 with two different sets of restriction enzymes and probing the digests with radio-labeled probes specific to the region of the mutation. Each digest gave the expected bands.

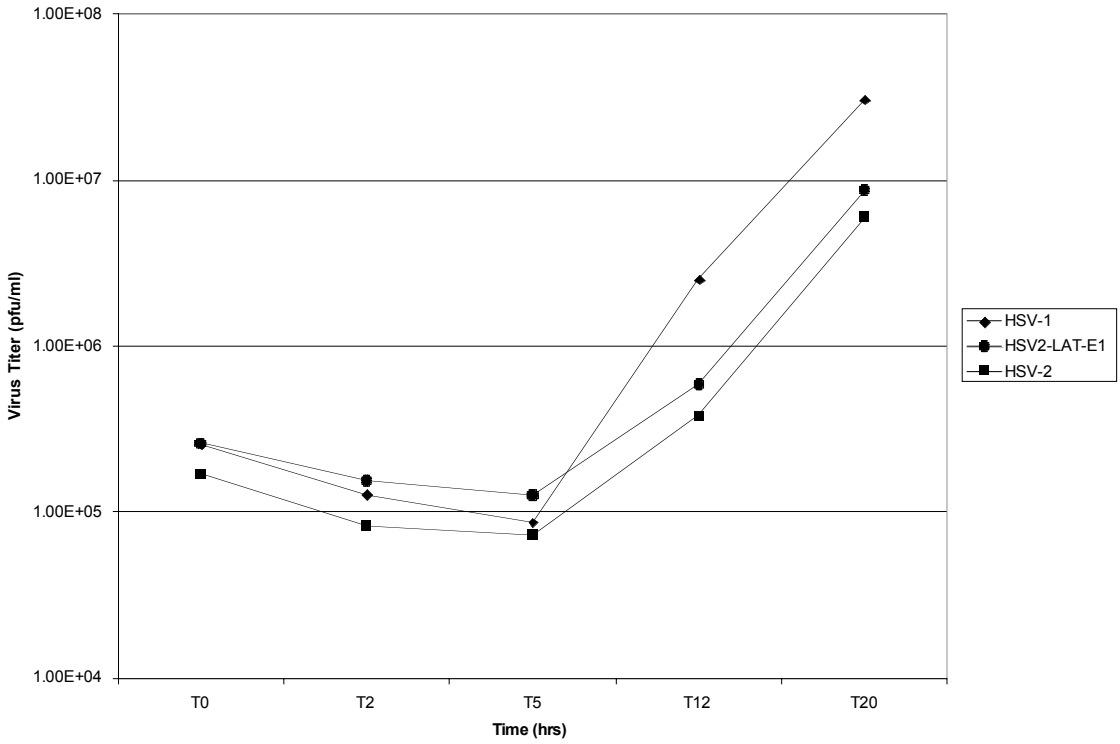


Figure 11. One-Step Growth Curves for HSV-2, HSV-1 and HSV2-LAT-E1.

To compare one-step growth characteristics of wild type and chimeric viruses, ~10^6 Vero cells were inoculated in duplicate at time 0 with a multiplicity of approximately 0.1 pfu/cell of each virus. Medium was added after a two-hour adsorption period. At 0, 2, 5, 12, and 20 hours post-inoculation, cells were scraped, freeze-thawed three times, and plaque-titered in duplicate. The wild type and chimeric viruses had comparable growth kinetics in cell culture.

Guinea Pig Studies

Female Hartley guinea pigs (Charles River, Wilmington, MA) were inoculated intravaginally or by injection into the right hind footpad with $2x10^5$ pfu of each virus. Guinea pigs were monitored and scored daily during acute infection (14 days) for lesion severity on a scale from 0 to 4: 0 = no disease, 1 = redness/swelling, 2 = 1-2 lesions, 3 = 3-5 lesions, 4 = 6 or more lesions or coalescence of lesions (Figures 12 and 13). Guinea pigs were also monitored daily for neurological symptoms in the form of hind limb weakness and urinary tract dysfunction. Hind limb weakness was assessed by placing the guinea pigs on a table in a normal resting position and extending their hind limbs behind them. Hind limb weakness was considered to be present if guinea pigs were unable to retract the hind limbs or if there was a minimum 3-second delay in retraction, even with gentle prodding. Urinary tract dysfunction was determined by presence of gross blood manually expressed from the bladder. Recurrences, defined as vesicular lesions, were enumerated during the latent phase from Day 15 through Day 42 or Day 60 p.i and graphed as cumulative recurrences per guinea pig in each group. Some studies were performed as acute phase studies in which guinea pigs were euthanized on Day 3 p.i. and/or Day 8 p.i., with a comparison group euthanized during latency. All observations of guinea pigs were made with the identity of the inoculated viruses masked. Animals were housed in American Association for Accreditation of Laboratory Animal Care approved facilities and cared for in accordance with institutional guidelines.

Lumbosacral dorsal root ganglia (DRG) and sacral and lumbar spinal cord tissues were collected from each animal immediately after sacrifice and snap frozen on dry ice. DNA and/or RNA were isolated from ganglia and spinal cord tissues using the Qiagen

DNA Minikit or the Qiagen AllPrep DNA/RNA Minikit (Valencia, CA) after homogenization with an Omni rotor-stator homogenizer with disposable tips (Omni International, Marietta, GA). The Qiagen DNA Minikit was used according to the manufacturer's protocol. The Qiagen AllPrep DNA/RNA Minikit protocol was used with the following modifications. DRG from the right side were pooled and homogenized in 600 ul RLT Buffer. Sacral and lumbar regions of the spinal cord were homogenized as whole segments in 20 ul RLT Buffer per mg of tissue. DNA/RNA was extracted from 300 ul of homogenate and the remainder was stored at -80°C. Homogenates were centrifuged for 6 minutes at maximum speed in a microcentrifuge and the supernatants were added to the DNA columns followed by 30 seconds centrifugation. Ethanol (300 ul 70%) was added to the flow-through, which was then placed into the RNA columns and centrifuged for 30 seconds. RNA columns were washed with 300 ul RQ Buffer. Fifteen ul Rnase-free Dnase in 65 ul RDD Buffer (Promega) was added to each column for in-column Dnase digestion for 20 minutes at room temperature, and columns were again washed with 300 ul RQ Buffer. RNA columns were washed twice with 500 ul RW Buffer, and RNA was eluted in 50 ul nuclease-free water. DNA columns were washed according to manufacturer's protocol and eluted in 100 ul EB Buffer. DNA and RNA were analyzed for concentration on a Nanodrop spectrophotometer. DNA was diluted to 10 ng/ul and RNA to 50 ng/ul.

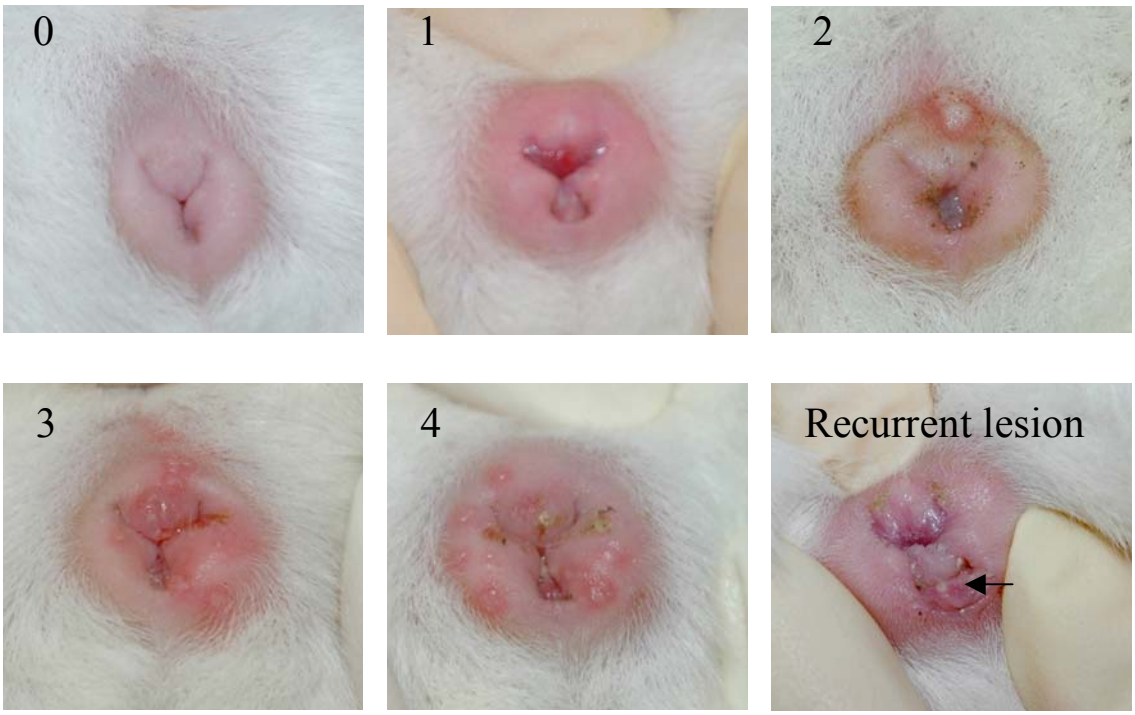


Figure 12. Lesion Severity Scoring in Genital Model.

Acute lesion severity is evaluated on a scale from 0 to 4. 0 = no symptoms, 1 = redness or swelling, 2 = 1-2 well-defined lesions, 3 = 3-5 lesions, 4 ≥ 6 lesions or coalescence of lesions. Typical lesions for each score are pictured here (score in upper left corner). Recurrent lesions are defined as vesicular lesions and can appear on the labia similar to acute lesions or just inside the rectal fold, as shown in the last photo.

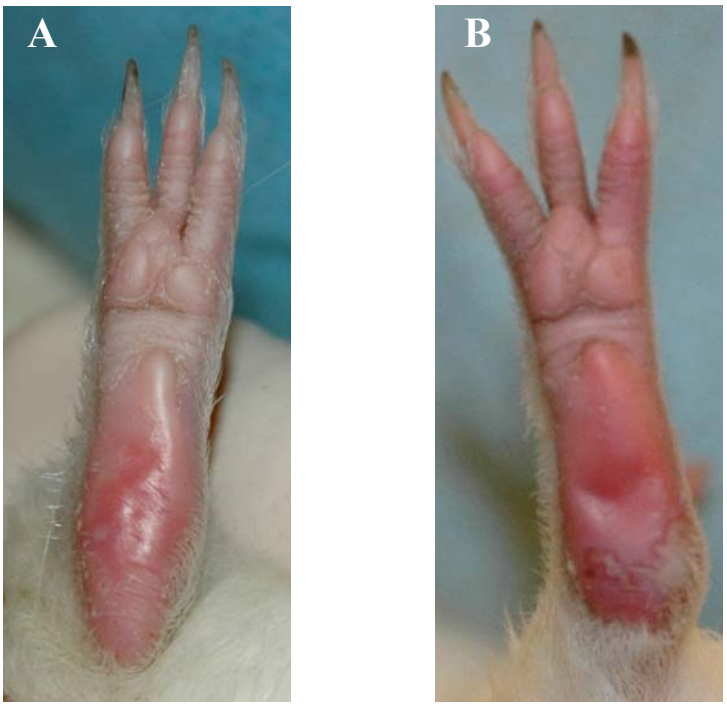


Figure 13. Lesion Severity Scoring in Footpad Model.

Typical lesions observed after footpad infection. Acute infection in the footpad model is typically less severe than in the genital model, so scoring is slightly modified. 0 = no symptoms, 1 = redness or swelling, 2 = 1 well-defined lesion, 3 = 2-3 lesions, 4 ≥ 4 lesions or coalescence of lesions. A) A single vesicular lesion on an erythematous base on the footpad. B) A severe infection with a severity score of 4 with coalescence of lesions and swelling on the heel.

Quantitative Real-Time PCR

The copy number of viral genomes and the quantity of guinea pig DNA and/or RNA in DRG and spinal cord were determined by quantitative real-time PCR using an Applied Biosystems Taqman PCR system, Model #7700 or 7900 (Applied Biosystems, Foster City, CA). Primers and probes were specific for sequences within HSV-2 and HSV-1 glycoproteins, LAT, ICP0, and thymidine kinase (TK) (Table 1). DNA was normalized to the 18S ribosome gene and RNA was normalized to the 18S ribosomal RNA using commercial primers and probe (Applied Biosystems, Foster City, CA).

PCR reactions contained Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), specific primers and probe in the concentrations given in Table 1, and 50 ng of DNA (5 ul of 10 ng/ul sample). Thermocycler conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec and 60°C for 1 min. RT-PCR reactions contained Taqman Universal RT-PCR One-Step Master Mix Cycle (Applied Biosystems, Foster City, CA), specific primers and probe in the concentrations given in Table 1, and 250 ng of RNA (5 ul of 50 ng/ul sample). Thermocycler conditions were 48°C for 30 min (RT step), 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec and 60°C for 1 min. A standard curve based on 10-fold dilutions of known amounts of plasmid or viral DNA was used to determine copy numbers for viral genes. Standards for normalization were made as 10-fold dilutions of DNA or RNA isolated from uninfected guinea pigs and quantified by spectrophotometry.

Table 1. Primers and Probes for Taqman Real-Time PCR Assays.

Primer/Probe	Sequence (5' – 3')	Concentration
HSV-2 glycoprotein D		
gD2 Forward	TCAGAGGATAACCTGGGA	250 nM
gD2 Reverse	GGGAGAGCGTACTTGCAGGA	250 nM
gD2 Probe	FAM-CCAGTCGTTTTCTTCACTAGCCGCAG-TAMRA	200 nM
HSV-2 LAT		
LAT2 Forward	GTCAACACGGACACACTCTTTTT	1600 nM
LAT2 Reverse	CGAGGCCTGTTGGTCTTTATC	1600 nM
LAT2 Probe	FAM-CACCCACCAAGACAGGGAGCCA-TAMRA	200 nM
HSV-2 TK		
TK2 Forward	TAATGACCAGCGCCCAGAT	900 nM
TK2 Reverse	CGATATGAGGAGCCAAAACG	900 nM
TK2 Probe	FAM-ACAATGAGCACGCCTTATGCGGC-TAMRA	250 nM
HSV-2 ICP0		
ICP02 Forward	GGTCACGCCCACTATCAGGTA	900 nM
ICP02 Reverse	CCTGCACCCCTTCTGCAT	900 nM
ICP02 Probe	FAM-CAACGGAATCCAGGTCTTCATGCACG-TAMRA	250 nM
HSV-1 glycoprotein G		
gG1 Forward	CTGTTCTCGTTCCTCACTGCCT	1000 nM
gG1 Reverse	CAAAAACGATAAGGTGTGGATGAC	1000 nM
gG1 Probe	FAM-CCCTGGACACCCTCTTCGTCGTCAG-TAMRA	250 nM
HSV-1 LAT		
LAT1 Forward	ACCCACGTACTCCAAGAAGGC	400 nM
LAT1 Reverse	TAAGACCCAAGCATAGAGAGCCA	400 nM
LAT1 Probe	FAM-TCCCACCCCGCCTGTGTTTTTGT-TAMRA	200 nM
HSV-1 TK		
TK1 Forward	AAAACCACCACCACGCAACT	900 nM
TK1 Reverse	TCATCGGCTCGGGTACGTA	900 nM
TK1 Probe	FAM-TGGGTTCGCGCGACGATATCG-TAMRA	250 nM
HSV-1 ICP0		
ICP01 Forward	GGATGCAATTGCGCAACAC	900 nM
ICP01 Reverse	GCGTCACGCCCACTATCAG	900 nM
ICP01 Probe	FAM-GCTGTGCAACGCCAAGCTGGTGTA-TAMRA	250 nM

Statistics

Statistics were performed on animal studies using nonparametric analyses with SPSS Version 11.5.0, including the Kruskal-Wallis and Mann-Whitney tests (LEAD Technologies). Comparisons of acute infections were based on total area-under-the-curve for lesion severity for days 1-14 after inoculation. Comparisons of recurrent infections were based on cumulative recurrences per guinea pig. Statistics on Taqman data were based on log-transformed quantities of DNA and RNA and analyzed by Student's t-test. Error bars represent standard error of the mean for each group.

Chapter 3

Herpes Simplex Virus Latency-Associated Transcript (LAT) Sequence Downstream of the Promoter Influences Type-Specific Reactivation and Viral Neurotropism

Introduction

During an initial infection, herpes simplex virus (HSV) establishes latency in the sensory nerve ganglia innervating the peripheral site of inoculation. In response to various stimuli, the virus can reactivate from the sensory neurons to cause recurrent disease at or near the original site of inoculation. HSV type 1 (HSV-1) reactivates preferentially from the trigeminal ganglia to cause recurrent orofacial herpes, while HSV type 2 (HSV-2) reactivates preferentially from the lumbosacral dorsal root ganglia (DRG) to cause recurrent genital herpes (53).

The trigeminal (TG) and dorsal root ganglion (DRG) sensory neurons in which HSV establishes latency have a single bifurcated axon, with one branch extending to the periphery and the second branch extending to the spinal cord. During primary infection or reactivation, the virus can reach the central nervous system (CNS) via the spinal cord branch of the axon. Central nervous system complications of infection may occur during either acute or recurrent infections. Encephalitis is more prevalent with HSV-1 and recurrent meningitis is more prevalent with HSV-2 (18, 47, 70, 96, 109), although the mechanism for this type-specific difference is not understood.

Previous studies have shown that the HSV latency-associated transcript (LAT) plays an important role in viral latency. HSV-1 and HSV-2 LAT deletion mutants are

impaired for recurrent disease (49, 55, 78). HSV-1 LAT is believed to enhance the establishment of latency, either by repression of lytic gene expression (22, 33, 61) or inhibition of apoptosis of neuronal cells (2, 13, 37, 45, 77, 81). Either of these mechanisms could influence neuronal cell survival, increasing the establishment of latency and providing a greater latent pool from which the virus could reactivate. While this may partially explain a role for LAT in reactivation in a general sense, the specific mechanism of type-specific reactivation of HSV-1 and HSV-2 is not understood.

We previously reported on a chimeric virus in which HSV-2 expressed the LAT from HSV-1, including the promoter and sequences extending to near the 3' end of the LAT intron (114). This chimeric virus, HSV-2/LAT1, exhibited a recurrence phenotype more similar to HSV-1 than to HSV-2, with a reduced reactivation frequency from the DRG in the guinea pig genital model and an increased reactivation frequency from TG in the rabbit eye model relative to wild-type HSV-2. Therefore, the LAT region encompassing the promoter, 5' exon, and intron provides the essential elements for type-specific reactivation of HSV-2.

To further define the region of LAT most important for HSV-2 type-specific reactivation, we constructed two additional chimeric HSV-2 viruses. We divided the region expressed in our previous HSV-2/LAT1 chimera into the promoter and sequence regions, and replaced these regions in HSV-2 with the corresponding sequences from HSV-1 (refer to Figure 5 on page 27). After *in vitro* characterization of the chimeric viruses, we tested the phenotype of these viruses in the guinea pig genital model of HSV infection. We also performed molecular studies of tissues from latently infected animals to evaluate differences in the distribution of viral DNA.

These studies demonstrate that the LAT sequence containing the 5' exon and the LAT intron contains the essential elements for type-specific reactivation of HSV-2. This LAT region also contributes to the virulence of the virus. We also provide evidence that HSV-1 and HSV-2 preferentially spread to different regions of the nervous system and that LAT influences the efficiency of HSV-2 replication in these different regions.

Results

Wild type and chimeric viruses produce similar acute lesion severity.

To determine whether the promoter or the sequence region of LAT provides the essential elements for type-specific reactivation of HSV-2, the viruses were evaluated in the guinea pig genital model. Female guinea pigs were inoculated intravaginally with 2 x 10^5 pfu of wild type HSV-2, wild type HSV-1, chimeric viruses HSV2-LAT-P1 and HSV2-LAT-S1, and the rescuant HSV2-LAT-S1-R. The severity of lesions was compared during the acute phase of infection through Day 14 post-inoculation (Figure 14). The mean lesion scores were similar between HSV2-LAT-S1, its rescuant and the wild type viruses. The acute infection with HSV2-LAT-P1 was less severe than wild type HSV-2 ($p=0.022$ by the Mann-Whitney test).

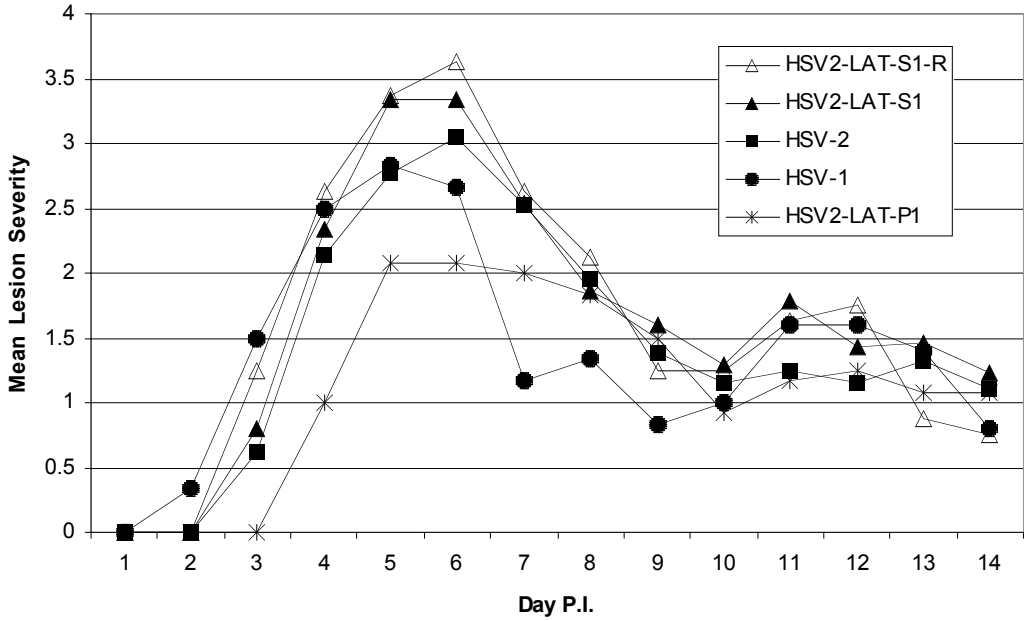


Figure 14. Acute Lesion Severity - HSV-2, HSV-1, HSV2-LAT-P1, HSV2-LAT-S1, HSV2-LAT-S1-R.

Lesion severity is graphed as the mean for each group of guinea pigs on each day of observation from Days 1-14 p.i., with 0 being no symptoms and 4 being the most severe. HSV-2 (n=21), HSV-1 (n=6), HSV2-LAT-P1 (n=12), HSV2-LAT-S1 (n=15), HSV2-LAT-D1-R (n=8). *The mean lesion scores were similar between HSV2-LAT-S1, its rescuant and the wild type viruses, while the acute infection with HSV2-LAT-P1 was less severe than wild type HSV-2. However, since we did not test the rescuant of HSV2-LAT-P1, we cannot definitively state that the reduced severity of HSV2-LAT-P1 was due to the defined LAT mutation.*

LAT sequence region contributes to autonomic nervous system involvement during acute infection.

During acute HSV infection, some guinea pigs experience urinary tract dysfunction, indicative of autonomic nervous system involvement. In the present study, the guinea pigs were evaluated for presence or absence of urinary tract dysfunction during the acute phase of infection. A significantly greater percentage of guinea pigs in the group infected with HSV2-LAT-S1 displayed urinary tract dysfunction during the acute phase of disease compared to its rescuant HSV2-LAT-S1-R ($p=0.035$), wild type HSV-2 ($p=0.004$), and HSV2-LAT-P1 ($p<0.001$) (Figure 15). Upon necropsy, bladders from guinea pigs infected with HSV2-LAT-S1 appeared hemorrhagic (Figure 16).

The LAT sequence, rather than the promoter, provides the essential elements for type-specific reactivation.

During the latent phase of infection, the reactivation frequency of HSV2-LAT-P1, the promoter mutant, was similar to wild type HSV-2 (Figure 17). In contrast, HSV2-LAT-S1, the LAT sequence mutant, reactivated inefficiently in the guinea pig genital model, similarly to wild type HSV-1 ($p=0.005$ compared to HSV-2 and $p=0.692$ compared to HSV-1). The rescuant HSV2-LAT-S1-R had a wild type HSV-2 reactivation phenotype. These data imply that the LAT sequence downstream of the PvuI restriction site, rather than the promoter region, provides the essential elements for type-specific reactivation of HSV-2.

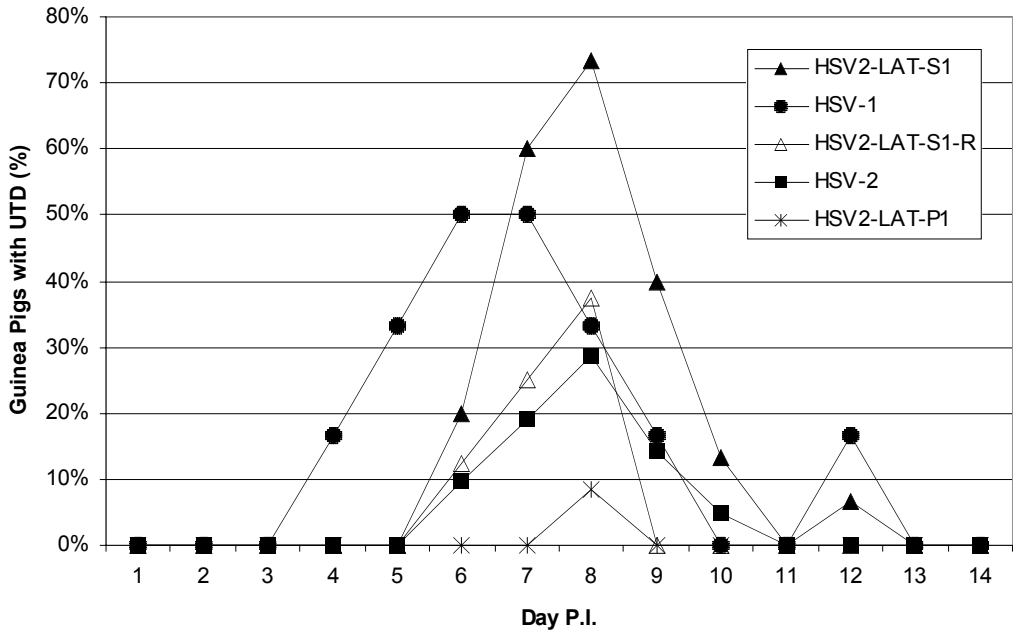


Figure 15. Urinary Tract Dysfunction - HSV-2, HSV-1, HSV2-LAT-P1, HSV2-LAT-S1, HSV2-LAT-S1-R

Percentages of guinea pigs from each group experiencing urinary tract dysfunction, indicative of autonomic nervous system involvement, Days 1-14 p.i. HSV-2 (n=21), HSV-1 (n=6), HSV2-LAT-P1 (n=12), HSV2-LAT-S1 (n=15), HSV2-LAT-S1-R (n=8). *A significantly greater percentage of guinea pigs in the group infected with HSV2-LAT-S1 displayed urinary tract dysfunction during the acute phase of disease compared to its rescuant HSV2-LAT-S1-R ($p=0.035$), wild type HSV-2 ($p=0.004$), and HSV2-LAT-P1 ($p<0.001$).*

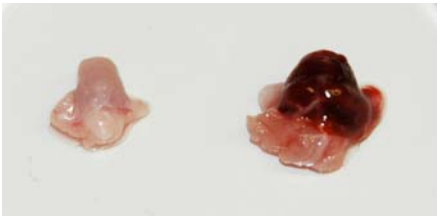


Figure 16. Urinary Bladder Involvement in HSV2-LAT-S1 infection.

Bladders of guinea pigs infected with HSV2-LAT-S1 appear hemorrhagic. Bladder on the left is a normal guinea pig bladder and bladder on the right is from a guinea pig infected with HSV2-LAT-S1. *Urinary bladders are affected to a much greater extent after HSV2-LAT-S1 infection compared to wild type HSV-2, suggesting that HSV2-LAT-S1 more efficiently spreads through the autonomic nervous system to establish a more destructive infection in pelvic organs.*

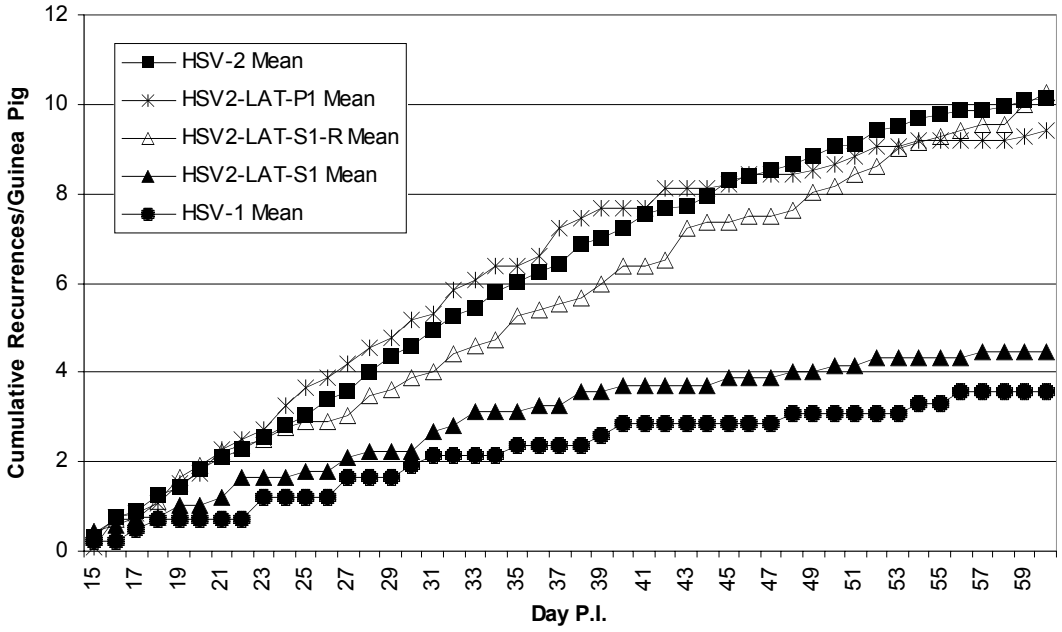


Figure 17. Cumulative Recurrences - HSV-2, HSV-1, HSV2-LAT-P1, HSV2-LAT-S1, HSV2-LAT-S1-R.

Cumulative recurrences per guinea pig for each group, adjusted for number of days of observation. HSV-2 (n=19), HSV-1 (n=5), HSV2-LAT-P1 (n=12), HSV2-LAT-S1 (n=13), HSV2-LAT-S1-R (n=8). Guinea pigs that did not survive acute disease were excluded. Significant p values are as follows: HSV-2 vs. HSV-1 = 0.023, HSV-2 vs. HSV2-LAT-S1 = 0.005, HSV-1 vs. HSV2-LAT-P1 = 0.011, HSV2-LAT-S1 vs. HSV2-LAT-P1 = 0.006. *These data imply that the LAT sequence downstream of the PvuI restriction site, rather than the promoter region, provides the essential elements for type-specific reactivation of HSV-2.*

LAT sequence region influences virulence during recurrences.

In this series of experiments, the HSV-2 virus expressing the HSV-1 LAT sequence unexpectedly demonstrated a greater mortality rate than the other viruses (Figure 18). Of the twenty guinea pigs inoculated with HSV2-LAT-S1, fifteen developed acute symptoms. Two of those fifteen died during the acute phase of infection, which is similar to the number observed with wild type HSV-2 (2 of 21), wild type HSV-1 (1 of 6), or the rescuant of HSV2-LAT-S1 (0 of 8). Eight of the remaining 13 guinea pigs died following recurrences, which were marked by severe and progressive clinical disease. This is in contrast to WT HSV-2 and the rescuant HSV2-LAT-S1-R, which typically caused mild recurrent peripheral lesions that resolved quickly (Kaplan-Meier $p<0.001$ compared to HSV-2 or HSV2-LAT-S1-R).

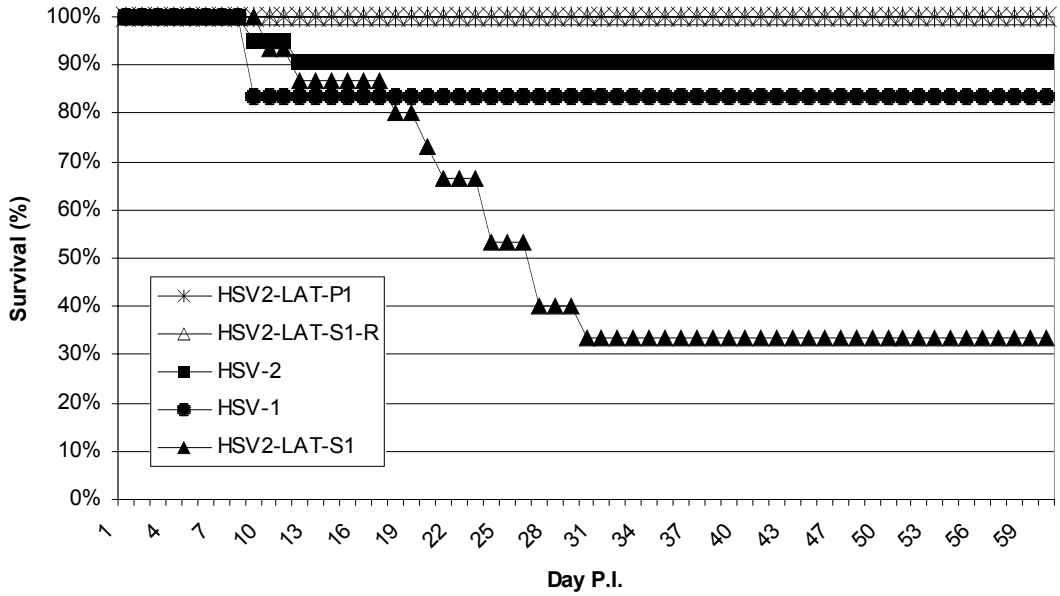


Figure 18. Survival of Guinea Pigs Infected with HSV-2, HSV-1, HSV2-LAT-P1, HSV2-LAT-S1, HSV2-LAT-S1-R.

Percentage of guinea pigs surviving acute and latent infection with HSV-2 (n=21), HSV-1 (n=6), HSV2-LAT-P1 (n=12), HSV2-LAT-S1 (n=15, and HSV2-LAT-S1-R (n=8). Acute infection is the first 14 days p.i. and latent infection begins on Day 15 p.i. After Day 15, guinea pigs died from recurrent disease. *HSV2-LAT-S1 infection results in severe, progressive, often fatal disease upon reactivation, suggesting that one function of the HSV-2 LAT region sequences is to limit the severity of disease to favor the establishment of latency.*

HSV-1 and HSV-2 spread efficiently to different regions of the nervous system.

To evaluate whether latent viral DNA load was responsible for the differences in reactivation, DNA was extracted from tissues harvested from the infected guinea pigs at the time of death. Viral DNA copy numbers were quantified by Taqman real-time PCR assay and normalized to the quantity of the gene for 18S ribosomal RNA (Figure 19). To evaluate differences between the viruses during latent infection and symptomatic recurrences, each group of infected guinea pigs was divided based on the absence or presence of symptoms at the time of tissue harvest. Comparisons during latent infection demonstrate differences in the distribution of the establishment of latency in neuronal tissues. One would expect virus to be actively replicating during symptomatic recurrences, thus differences between viral DNA loads in animals whose tissues were harvested during symptomatic recurrences would provide an indication of the efficiency of replication in the various regions of the nervous system after viral reactivation.

In asymptomatic latently infected guinea pigs (Figure 19A), viral DNA levels were similar between all of the HSV-2 viruses, with the largest quantities of viral DNA found in the sacral spinal cord. However, HSV-1 DNA was primarily found in the lumbar spinal cord ($p<0.0005$ compared to all of the HSV-2 viruses), suggesting that HSV-1 is capable of establishing latency in the lumbar cord while HSV-2 is capable of establishing latency in the sacral cord in addition to the DRG.

In guinea pigs with symptoms at the time of tissue extraction, there was no significant difference between the viral DNA copy numbers of HSV-1 and HSV-2 in the DRG ($p=0.527$) (Figure 19B). However, DNA of all of the wild type and chimeric HSV-2 viruses was present at significantly higher levels in the sacral spinal cord as compared

to HSV-1 ($p<0.022$). HSV-1 DNA was primarily found in the lumbar spinal cord ($p<0.0005$ compared to the HSV-2 viruses). Thus, HSV-1 and HSV-2 replicate more efficiently in different regions of the central nervous system during symptomatic recurrences, which may provide further insight into the mechanism for type-specific reactivation of HSV-1 and HSV-2 and into different patterns of CNS infection. During recurrences, HSV-2 viral DNA quantities increased in the sacral spinal cord and the DRG when compared to levels present in asymptomatic animals, while HSV-1 DNA quantities increased in the lumbar cord and the DRG (compare Figures 19A and 19B), which suggests involvement of the spinal cord during reactivation of both HSV-1 and HSV-2.

LAT sequence influences the neurotropism of HSV-2.

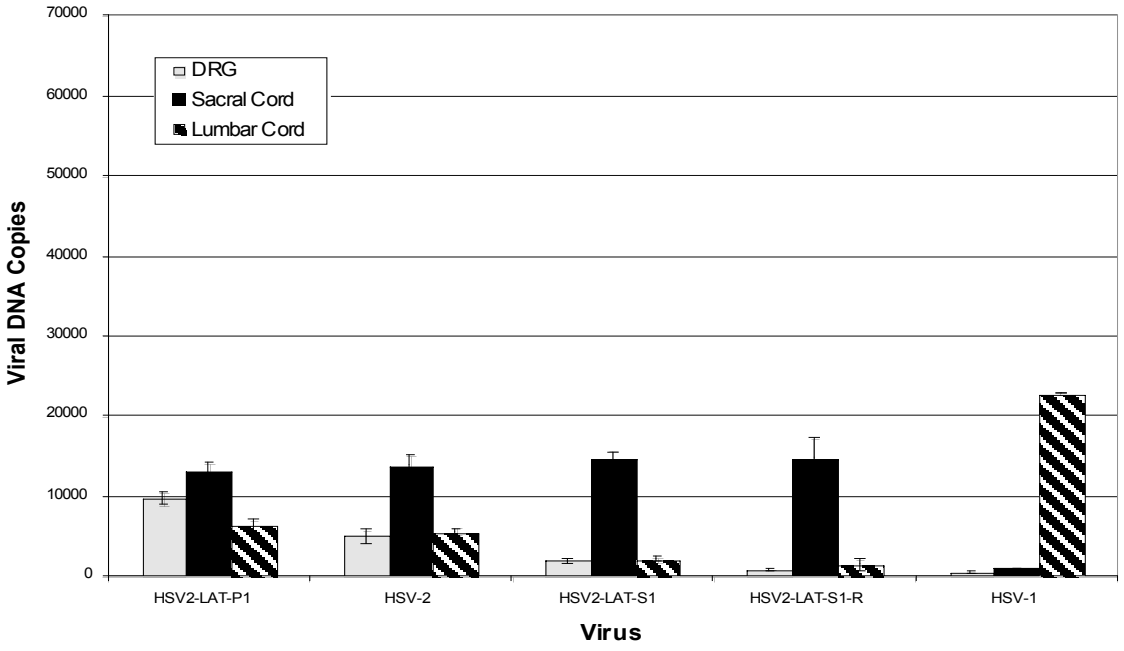
During latent infection (Figure 19A), the viral DNA quantities were similar between HSV2-LAT-S1, its rescuant, HSV2-LAT-P1 and wild type HSV-2 in the DRG, the sacral spinal cord, and the lumbar spinal cord. During recurrences, guinea pigs infected with HSV2-LAT-S1 had less viral DNA in their DRG when compared with animals infected with wild type HSV-2 ($p=0.021$), although HSV2-LAT-S1 was not significantly different from the rescuant ($p=0.513$) (Figure 19B), implying that differences in the DRG may not have a significant impact on the behavior of the virus. In the sacral spinal cord, animals infected with the more virulent HSV2-LAT-S1 produced higher levels of viral DNA compared to wild type HSV-2 and the rescuant HSV2-LAT-S1-R during periods of active viral replication ($p=0.007$ compared to either HSV-2 or HSV2-LAT-S1-R) (Figure 19B). These data suggest that the LAT sequence contributes to the ability of the virus to either spread to or replicate in different regions of the nervous

system during reactivations, although the LAT sequences are not the sole determinants of viral spread.

Figure 19. Viral DNA Quantities of HSV-2, HSV-1, HSV2-LAT-P1, HSV2-LAT-S1, and HSV2-LAT-S1-R in the DRG, Sacral Spinal Cord, and Lumbar Spinal Cord.

Quantities of viral DNA in the DRG, sacral spinal cord, and lumbar spinal cord, quantified by Taqman PCR assay and normalized to the gene for the 18S ribosomal RNA. 19A) Viral DNA copy numbers quantified in guinea pig tissues during latent infection with no observable symptoms. HSV-1 differs significantly from all HSV-2-based viruses in lumbar cord ($p<0.0005$). HSV2-LAT-P1 (n=12), HSV-2 (n=13), HSV2-LAT-S1 (n=7), HSV2-LAT-S1-R (n=2), HSV-1 (n=3). 19B) Viral DNA copy numbers quantified in guinea pig tissues obtained during symptomatic recurrences. HSV-1 differs significantly from HSV-2 viruses in sacral cord ($p<0.022$) and in lumbar cord ($p<0.0005$). HSV2-LAT-S1 differs significantly in the DRG from HSV-2 ($p=0.021$) and in the sacral cord from HSV-2 ($p=0.007$), HSV2-LAT-S1-R ($p=0.007$), and HSV-1 ($p<0.0005$). HSV2-LAT-P1 (no tissues extracted during symptomatic recurrences), HSV-2 (n=5), HSV2-LAT-S1 (n=5), HSV2-LAT-S1-R (n=4), HSV-1 (n=2). *HSV-2 and HSV-1 spread more efficiently to different regions of the central nervous system where they preferentially establish latency, and the viruses replicate more efficiently in different regions of the central nervous system during symptomatic recurrences, suggesting involvement of the spinal cord during reactivation. The LAT sequence contributes to the ability of the virus to either spread to or replicate in different regions of the nervous system, although the LAT is not the sole determinant of spread.*

A


70000
60000
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DRG
Sacral Cord
Lumbar Cord
Viral DNA Copies
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HSV2-LAT-S1-R
HSV-1
Virus

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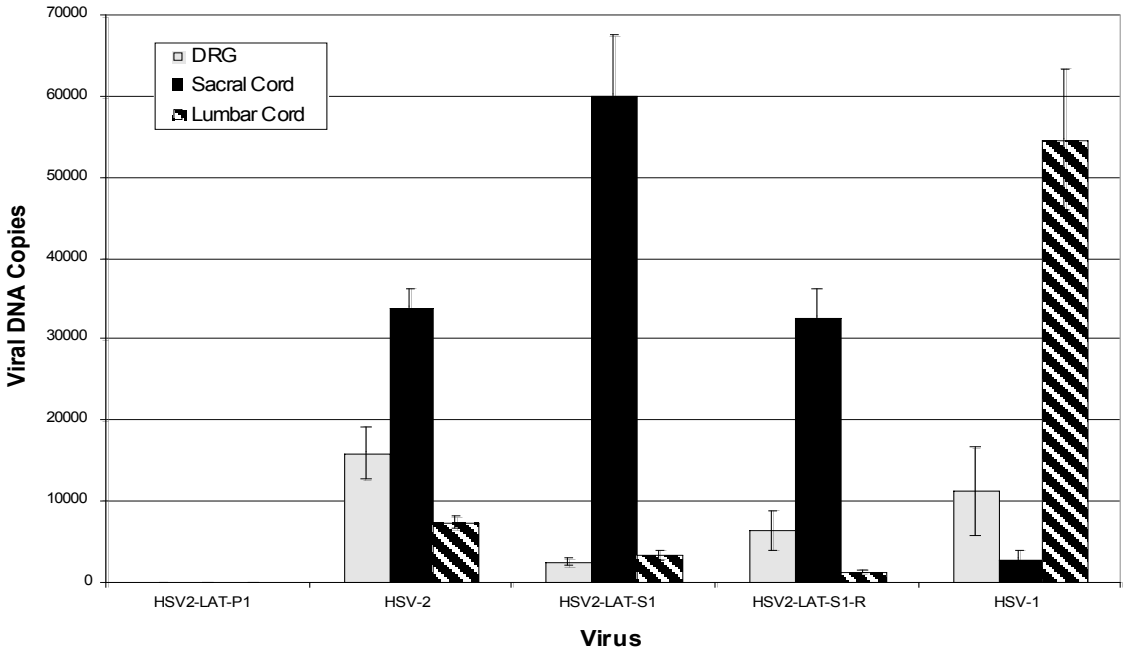
70000
60000
50000
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DRG
Sacral Cord
Lumbar Cord
Viral DNA Copies
HSV2-LAT-P1
HSV-2
HSV2-LAT-S1
HSV2-LAT-S1-R
HSV-1
Virus

Discussion

Previously in our lab, a chimeric HSV-2 virus expressing the LAT from HSV-1 failed to reactivate efficiently in the guinea pig genital model of HSV infection (114). One goal of the current study was to determine whether the LAT promoter or the LAT sequence provides the essential elements for type-specific reactivation of HSV-2. The HSV LAT region substituted in our previous chimera was divided into the promoter region (from NotI to PvuI) and the sequence region (from PvuI to XhoI), and additional chimeric HSV-2 viruses were constructed. HSV2-LAT-P1 is HSV-2 that expresses the native HSV-2 LAT sequence under the control of the HSV-1 LAT promoter. HSV2-LAT-S1 is HSV-2 that expresses HSV-1 LAT sequences, including the 5' exon and most of the LAT intron, under the control of the native HSV-2 LAT promoter. HSV2-LAT-P1 reactivated efficiently in the guinea pig genital model of infection, although the acute infection was attenuated in both lesion severity and urinary tract dysfunction. The chimeric HSV2-LAT-S1 virus and its rescuant produced acute infections with lesion severities similar to wild type HSV-2 as well as wild type HSV-1. However, HSV2-LAT-S1 reactivated inefficiently in the guinea pig genital model of infection, with a reactivation frequency similar to that of wild type HSV-1, and a wild type recurrence phenotype was restored with its rescuant HSV2-LAT-S1-R. Thus the LAT sequence including the 5' exon and intron, rather than the LAT promoter, provides the essential elements for efficient genital reactivation of HSV-2. This correlates well with studies on HSV-1, which imply that the region between the promoter and the LAT intron contains the reactivation critical region (10, 40, 62) and enhancer activities (5, 57) for HSV-1. Since this region of HSV-1 has known enhancer activity, we hypothesize that enhancer

activity within this region of both HSV-1 and HSV-2 may play a role in determining site-specific recurrence phenotypes.

Infections with the HSV2-LAT-S1 chimera were characterized by a dramatic increase in virulence and mortality relative to wild type HSV-2, the rescuant HSV2-LAT-S1-R, HSV2-LAT-P1 and wild type HSV-1. During acute disease, a greater percentage of animals infected with HSV2-LAT-S1 developed urinary tract dysfunction compared to HSV-2, suggestive of more pronounced autonomic nervous system involvement. During latency, HSV-1 and HSV2-LAT-S1 failed to efficiently reactivate. During its rare recurrences, HSV-1 produced a single vesicular lesion lasting 1-2 days, similar in character to recurrences produced by HSV-2, HSV2-LAT-P1, and HSV2-LAT-S1-R. However, in guinea pigs infected with the HSV2-LAT-S1 chimera, recurrent disease progressed from a single lesion to multiple lesions coinciding with neurological symptoms, followed by rapid deterioration and death. The most likely explanation for this progression of symptoms is that the HSV2-LAT-S1 chimera was able to spread more efficiently through the nervous system via a change in neurotropism, mediated by its HSV-1 LAT sequences. In a previous report on HSV-1 LAT region deletion mutants, virulence either increased or decreased depending on the size of the deletion and the species infected (79), which suggests that the LAT region has multiple functional elements and that specific neuronal factors may interact with LAT sequences in HSV-1 to regulate viral replication. Fatal recurrences were not observed in the previously tested HSV-2/LAT1 chimeric virus, which contained both HSV-1 LAT promoter and sequence, implying that an interaction between the HSV-2 LAT promoter and HSV-1 LAT sequence may have permitted unusual spread in the HSV2-LAT-S1 infection,

contributing to its increased virulence. Alternatively, a *cis*-acting regulatory element located at the mutation junction site may have been disrupted, permitting unusual replicative characteristics. Although the LAT promoters are fairly well conserved, there is little discernible similarity between the HSV-1 and HSV-2 LAT regions downstream of the promoter. This region in both HSV-1 and HSV-2 is densely populated by putative transcription factor binding sites and enhancer elements, which likely function cooperatively to modulate the activity of the LAT region. LAT sequences could play a role in down-regulation of viral replication, which may fail when non-type-specific sequences are introduced or when specific sequences are deleted, or these LAT sequences could facilitate growth in different types of neurons, which could also give rise to an unusual neurotropic phenotype.

The acute infection caused by HSV2-LAT-P1 was attenuated in both lesion severity and urinary tract dysfunction. However, since HSV2-LAT-P1 demonstrated a wild type reactivation phenotype, a rescuant of this chimeric virus was not constructed. Any secondary mutation that may have occurred during the construction of the chimera did not affect the reactivation phenotype, although construction of a rescuant would be necessary to ascertain whether the modest differences observed during acute infection of HSV2-LAT-P1 compared to HSV-2 were attributable to the LAT promoter substitution.

HSV-1 spread to the lumbar spinal cord more efficiently than HSV-2, while all of the HSV-2 based viruses spread more efficiently than HSV-1 to the sacral spinal cord. The autonomic nervous system innervates the genitalia and bladder through both sympathetic and parasympathetic fibers extending from the paracervical ganglia to the pelvic organs. Pre-synaptic parasympathetic fibers extend from the sacral spinal cord to

the paracervical ganglia while sympathetic fibers originate in the lumbar cord (42, 72, 73). While HSV-1 has been shown to replicate and establish latency in sympathetic neurons (34, 51, 52, 101), HSV-2 appears to be limited in its ability to replicate or establish latency in sympathetic neurons (75). HSV-1 and HSV-2 are both able to gain access to parasympathetic (15, 75, 102) and sensory nerve fibers. It is thus possible that the difference between lumbar and sacral quantities of HSV-1 and HSV-2 DNA could be related to different autonomic pathways for reaching the cord, potentially more likely to be sympathetic for HSV-1 and parasympathetic for HSV-2. During recurrences, HSV-2 viral DNA quantities increased in both the sacral cord and the DRG when compared with levels present during latent infection (compare Figure 19A to 19B), suggesting that HSV-2 viral replication may occur in both the sacral cord and the DRG during reactivation. HSV-1 exhibited increases in viral DNA in both the lumbar cord and the DRG during recurrences, suggesting that HSV-1 may also replicate in the lumbar spinal cord as well as the DRG during reactivation. However, it is unclear whether the increased DNA in the spinal cord during symptomatic recurrences is the result of viral spread from the DRG or reactivation originating in neurons of the spinal cord. Since genital reactivation of viruses with greater quantities of viral DNA in the sacral cord was more efficient, this also raises the possibility that some proportion of HSV reactivation may occur through autonomic pathways, which may provide further insight into type-specific differences in reactivation.

Substitution of HSV-1 LAT sequences into the HSV-2 LAT region did not eliminate the HSV-2 preference for the sacral spinal cord, but did alter the quantity of DNA found in the sacral cord and the DRG during recurrences. The more virulent

sequence chimera, HSV2-LAT-S1, had higher levels of viral DNA in the sacral cord and lower levels in the DRG as compared to wild type HSV-2 during recurrences. Thus, the LAT sequence is not the major factor that permits HSV-2-based viruses to more efficiently reach the sacral spinal cord but it does influence this process, potentially via a mechanism involving spread through or replication in autonomic neurons. The increased efficiency of HSV2-LAT-S1 in reaching and replicating in the sacral spinal cord further supports the conclusion that LAT region sequences are involved in neurotropism, or the ability of the virus to infect and replicate within specific neuronal subtypes.

Consistent with previous experiments, HSV-1 failed to reactivate efficiently in the guinea pig genital model. Wild type HSV-2, HSV2-LAT-P1, and HSV2-LAT-S1-R reactivated efficiently, and these viruses also showed relatively higher DNA levels in the sacral spinal cord compared to HSV-1. This suggests that efficient replication in the sacral spinal cord, and the ability to appropriately down-regulate viral replication, is correlated with efficient genital reactivation during latency. Although the HSV2-LAT-S1 chimera efficiently reached the sacral spinal cord, its reduced recurrence phenotype and high degree of virulence after reactivation may have been due to an inability to down-regulate the productive cycle upon reactivation, thus destroying its host instead of re-establishing a latent state from which it could again reactivate.

HSV-1 and HSV-2, while similar in many ways, exhibit significant differences in patterns of latency and reactivation, manifested clinically by site-specific recurrence patterns and differences in CNS manifestations. In the present study, we show that type-specific recurrence of HSV-2 depends on the presence of appropriate LAT-region sequences downstream of the promoter. This same region of the LAT sequence appears

to influence neurotropism within the central nervous system as well as the autonomic nervous system, suggesting that some portion of the LAT phenotype may be due to an effect on viral replication and/or establishment of latency in different types of neurons.

Chapter 4

Herpes Simplex Virus Type 2 Latency-Associated Transcript (LAT) Exon 1 Confers Type-Specific Reactivation

Introduction

Many studies have attempted to determine the specific region of LAT that is responsible for reactivation *in vivo* (6, 10, 29, 38, 39, 44, 45, 59, 78, 80, 83, 85, 100, 114), although only two of these studies included comparisons with HSV-2 (38, 114). Recombinant HSV-1 viruses with deletions in the LAT region have mapped the reactivation phenotype to the first 1.5 kb of LAT in HSV-1, including the promoter region, exon 1, and the LAT intron (80, 87). HSV-1 and HSV-2 LAT promoter mutants, which express no detectable LAT during latency, are impaired for reactivation both spontaneously and after induction in several animal models (9, 17, 23, 49, 64). The LAT intron expressed transgenically in mice has no influence on HSV infection or reactivation phenotype (106) and recombinant viruses with deletions in the intron behave like wild type virus (30). Mutation of the splice branch points destabilizes accumulation of the LAT intron but does not influence reactivation of HSV-1 (71, 74). Smaller deletions within this 1.5 kb region in HSV-1 suggest that the sequence between the promoter and the 5' splice site of the LAT intron contains essential elements for reactivation of HSV-1 (10, 39). Deletions within HSV-1 exon 1 also alter virulence differently in mice and rabbits (79), suggesting that this region may respond to cellular factors to exert an effect on virulence. LAT exon 1 also provides enhancer functions for continued long-term expression of HSV-1 LAT during latency (5, 57). Thus, the LAT exon 1 may confer regulatory influence over reactivation and virulence.

Previous work with chimeric viruses HSV2-LAT-P1 (promoter chimera) and HSV2-LAT-S1 (sequence chimera) provided evidence that the critical elements for type-specific reactivation of HSV-2 reside in the region between the Pvu I restriction site just downstream of the TATA box and an Xho I restriction site within the LAT intron. To further define the region of LAT critical for HSV-2 type-specific reactivation, an additional chimeric virus was constructed. The region of HSV-2 from the LAT TATA box to the 5' splice site of the LAT intron was replaced by the corresponding region of HSV-1, and designated HSV2-LAT-E1 (for Exon from HSV-1). A rescuant, HSV2-LAT-E1-R, was also constructed to restore the native HSV-2 sequences. After *in vitro* characterization of HSV2-LAT-E1, the chimeric virus and its rescuant were tested for reactivation in the guinea pig genital model of HSV infection. Tissues were also evaluated for biodistribution of viral DNA to determine if LAT exon 1 influenced viral spread and the establishment of latency. To evaluate a potential mechanism for any differences, viral transcript expression levels were quantified for LAT, ICP0 (a viral transactivator essential for reactivation), and thymidine kinase (tk, which is transcribed during active replication).

These studies demonstrate that the LAT sequence that lies between the TATA box and the 5'splice site of the LAT intron (LAT exon 1) contains the essential elements for type-specific reactivation of HSV-2. However, LAT exon 1 has no significant effect on the viral DNA load or expression of LAT, ICP0, or thymidine kinase transcripts in the DRG or regions of the spinal cord.

Results

HSV2-LAT-E1 produces wild type HSV-2 acute lesion severity.

To determine if the LAT exon 1 provides the essential elements for reactivation of HSV-2, wild type HSV-2, chimeric virus HSV2-LAT-E1, and its rescuant HSV2-LAT-E1-R were evaluated in the guinea pig genital model. Female guinea pigs were inoculated intravaginally with 2×10^5 pfu of virus. The severity of lesions was compared during the acute phase of infection through Day 14 post-inoculation (Figure 20). The mean lesion scores were similar between HSV2-LAT-E1, its rescuant HSV2-LAT-E1-R, and wild type HSV-2 ($p=0.536$ according to the Kruskal-Wallis Test).

LAT exon 1 contributes to autonomic nervous system involvement during acute infection.

Compared to wild type HSV-2 or HSV2-LAT-E1-R, a greater percentage of the guinea pigs infected with HSV2-LAT-E1 displayed symptoms of autonomic nervous system involvement in the form of bladder dysfunction during the acute phase of infection (Kruskal-Wallis $p=0.011$) (Figure 21). 68% of the guinea pigs infected with HSV2-LAT-E1 experienced bladder dysfunction compared to 38% of those infected with wild type HSV-2 and 23% of those infected with the rescuant HSV2-LAT-E1-R.

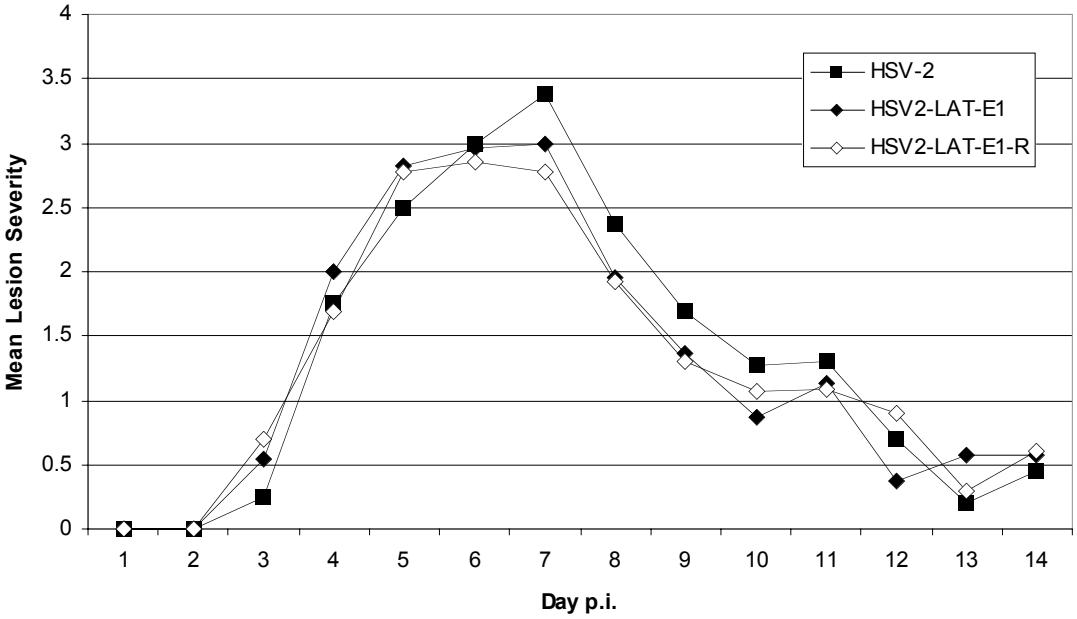


Figure 20. Acute Lesion Severity - HSV-2, HSV2-LAT-E1, HSV2-LAT-E1-R

Lesion severity is graphed as the mean for each group of guinea pigs on each day of observation from Days 1-14 p.i., with 0 being no symptoms and 4 being the most severe. HSV-2 (n=19), HSV2-LAT-E1 (n=25), HSV2-LAT-E1-R (n=13). Kruskal-Wallis p=0.515. *HSV2-LAT-E1 produced an acute infection similar in severity to HSV-2 and the rescuant HSV2-LAT-E1-R.*

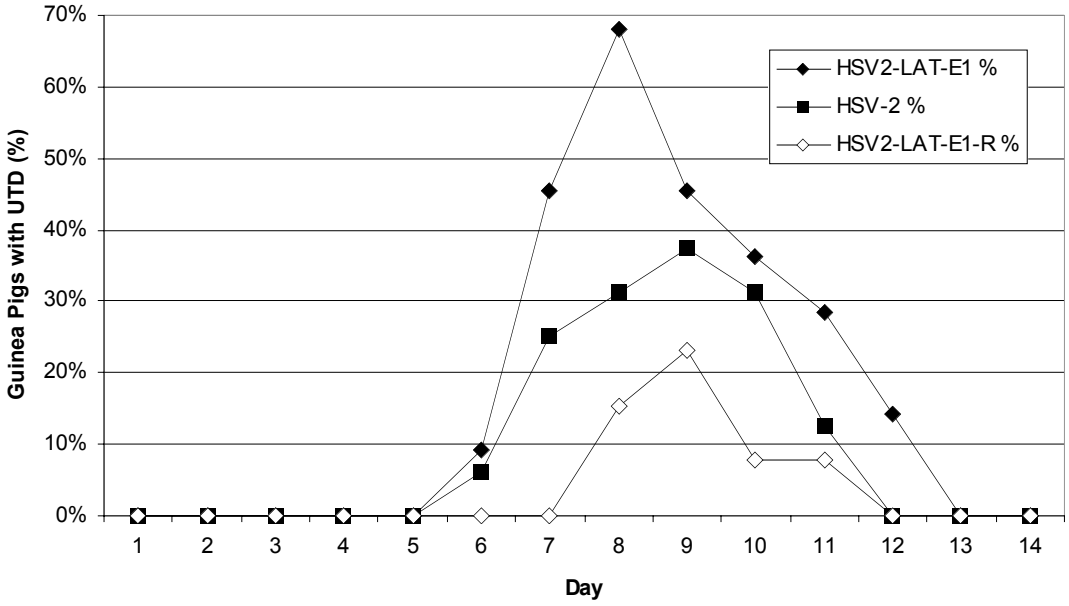


Figure 21. Urinary Tract Dysfunction - HSV-2, HSV2-LAT-E1, HSV2-LAT-E1-R.

Percentages of guinea pigs from each group experiencing urinary tract dysfunction, indicative of autonomic nervous system involvement, Days 1-14 p.i. HSV-2 (n=19), HSV2-LAT-E1 (n=25), HSV2-LAT-E1-R (n=13). *A greater percentage of guinea pigs infected with HSV2-LAT-E1 displayed symptoms of autonomic nervous system involvement in the form of bladder dysfunction during the acute phase of infection, suggesting that HSV2-LAT-E1 gained the ability to spread more efficiently through autonomic pathways to the bladder.*

LAT exon 1 confers the type-specific recurrence phenotype of HSV-2.

During the latent phase of infection, HSV2-LAT-E1 reactivated inefficiently in the guinea pig genital model compared to wild type HSV-2 ($p<0.0005$) or the rescuant HSV2-LAT-E1-R ($p=0.001$) (Figure 22). The rescuant HSV2-LAT-E1-R had a wild type recurrence phenotype ($p=0.221$ compared to HSV-2). These results demonstrate that the LAT exon 1 provides the essential elements for type-specific reactivation of HSV-2.

LAT exon 1 does not significantly affect viral load in the DRG or spinal cord.

To determine if LAT exon 1 influences reactivation by affecting viral spread and the establishment of latency, tissues from infected guinea pigs were evaluated for viral DNA levels in the DRG and lumbosacral spinal cord. Guinea pigs were sacrificed on Day 8 and Day 42 post-inoculation to evaluate differences between acute and latent viral load. During acute infection, HSV2-LAT-E1 had significantly higher levels of viral DNA in the lumbar spinal cord compared to its rescuant ($p=0.028$), but not significantly higher than wild type HSV-2 ($p=0.10$) (Figure 23). HSV2-LAT-E1 DNA was also significantly decreased in the lumbar spinal cord during latency compared to HSV-2 ($p=0.050$) although the decrease was not significantly different from the rescuant. These results likely reflect variability among the guinea pigs and are not likely to be biologically relevant to reactivation competence. Although viral DNA levels of HSV2-LAT-E1 were decreased in the DRG compared to wild type HSV-2 during latency ($p=0.025$), the rescuant HSV2-LAT-E1-R was also significantly decreased compared to wild type HSV-2 ($p=0.031$). Since HSV2-LAT-E1-R reactivated with wild type kinetics

and HSV2-LAT-E1 did not reactivate efficiently, these data suggest that viral load in the DRG does not affect reactivation competence.

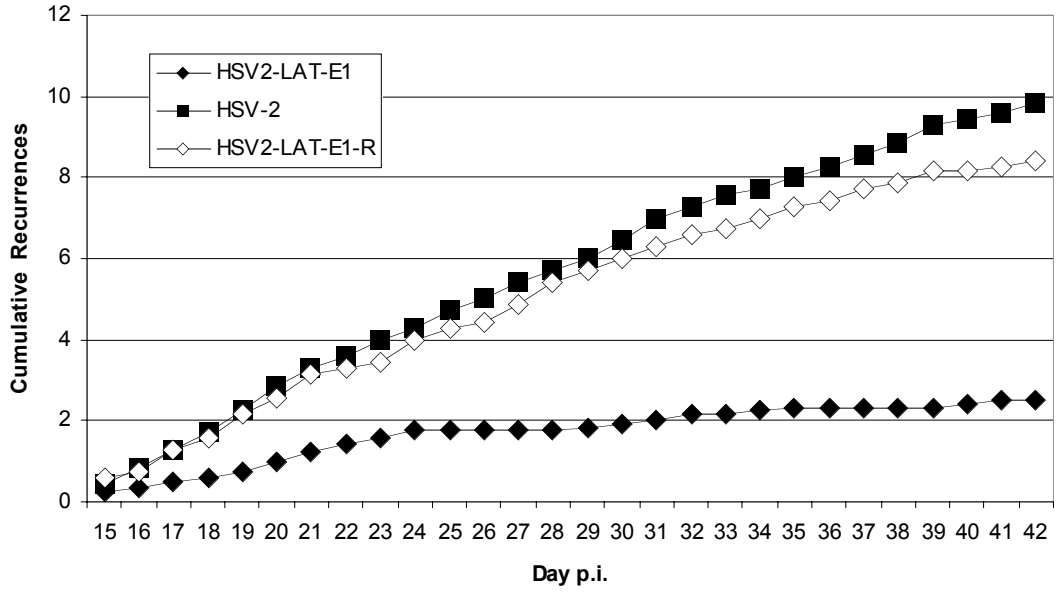
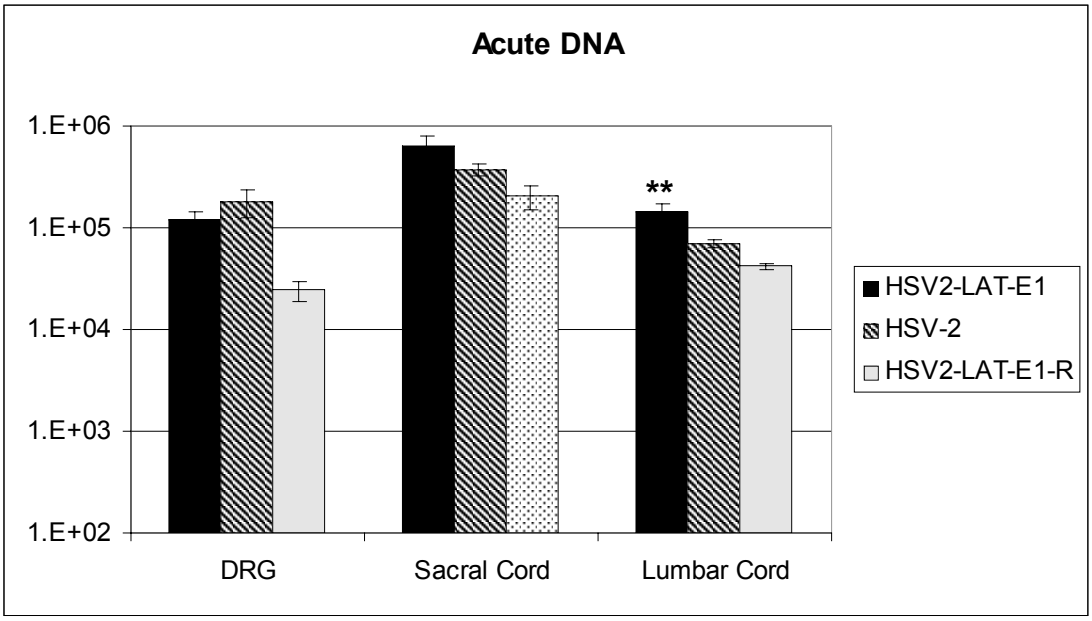


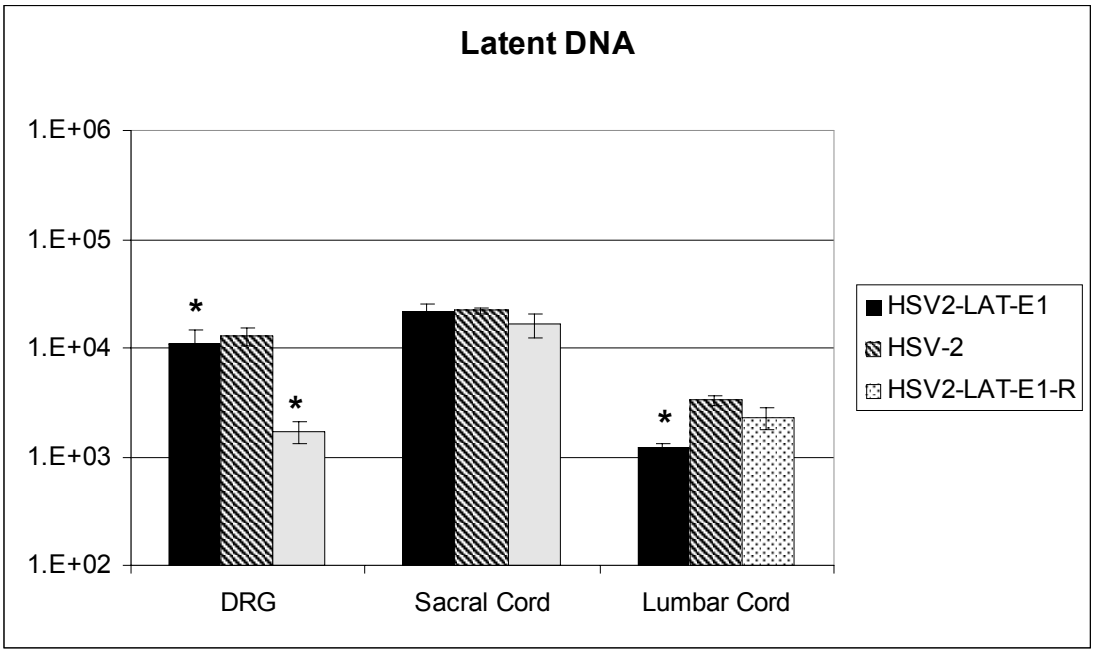
Figure 22. Cumulative Recurrences - HSV-2, HSV2-LAT-E1, HSV2-LAT-E1-R.

Cumulative recurrences per guinea pig for each group during latent infection. HSV-2 (n=7), HSV2-LAT-E1 (n=13), HSV2-LAT-E1-R (n=7). Guinea pigs that did not survive acute disease were excluded. Mann-Whitney p values are as follows: HSV-2 vs. HSV2-LAT-E1 < 0.0005, HSV2-LAT-E1 vs. HSV2-LAT-E1-R = 0.001, HSV-2 vs. HSV2-LAT-E1-R = 0.221. *LAT exon 1 provides the essential elements for type-specific reactivation of HSV-2.*

Figure 23. Viral DNA Quantities of HSV-2, HSV2-LAT-E1, and HSV2-LAT-E1-R in the DRG, Sacral Cord, and Lumbar Cord during Acute and Latent Infection.

Viral DNA extracted from the lumbosacral DRG, sacral spinal cord, and lumbar spinal cord during acute infection (Day 8 p.i.) and latent infection (Day 42 p.i.), quantified by Taqman PCR assay and normalized to the gene for the 18S ribosomal RNA. * $p<0.05$ compared to HSV-2. ** $p<0.05$ compared to rescuant HSV2-LAT-E1-R. Acute: HSV2-LAT-E1 (n=11), HSV-2 (n=10), HSV2-LAT-E1-R (5). Latent: HSV2-LAT-E1 (n=14), HSV-2 (n=9), HSV2-LAT-E1-R (n=8). *LAT exon 1 does not significantly affect viral DNA load, therefore viral DNA load in the DRG does not correlate with reactivation competence.*


Acute DNA
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
**
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Latent DNA
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
*
*
*
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R

LAT exon 1 does not significantly affect LAT, ICP0, or thymidine kinase transcript expression.

To better understand the mechanism by which the LAT exon 1 may regulate reactivation of HSV-2, the viral transcripts for LAT, ICP0, and thymidine kinase (*tk*) were quantified by Taqman quantitative RT-PCR assays. Guinea pigs were sacrificed during acute infection (Day 8 p.i.) and latent infection (Day 42 p.i.) to evaluate differences in gene expression between acutely and latently infected animals (Figure 24).

During acute infection, the only significant difference between the viruses was HSV2-LAT-E1 expression of LAT in the lumbar cord. This difference was narrowly significant compared to HSV-2 ($p=0.048$) but was not statistically different from the rescuant HSV2-LAT-E1-R ($p=0.914$), suggesting that this result is not relevant to reactivation. Decreased LAT expression and increased viral DNA quantities during acute infection suggest that HSV2-LAT-E1 may have a slight growth advantage in the lumbar cord compared to the wild type viruses, although a firm conclusion cannot be drawn without definitive statistical differences between the chimeric virus and both the wild type HSV-2 and the rescuant.

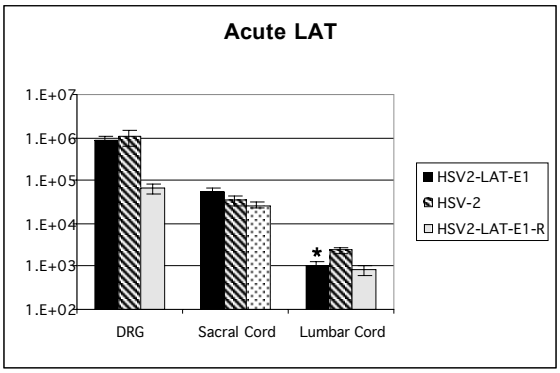
During latency, there was a modest decrease in LAT expression by HSV2-LAT-E1-R in the DRG ($p=0.043$) and by HSV2-LAT-E1 in the lumbar spinal cord ($p=0.045$) compared to HSV-2. These results are likely due to animal variability since HSV2-LAT-E1 LAT expression levels in the lumbar cord were similar to its rescuant ($p=0.567$), which was not statistically different from HSV-2 ($p=0.057$).

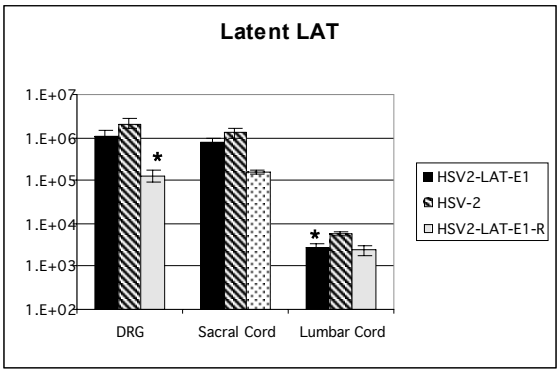
HSV2-LAT-E1-R expressed significantly less thymidine kinase (*tk*) during latent infection compared to both wild type HSV-2 ($p=0.011$) and HSV2-LAT-E1 ($p=0.049$). The *tk* gene is only expressed during replication of the virus, so expression during latency represents a reactivation. Although animals are sacrificed when no lesions are observed, it is possible that a reactivation may be occurring but is either asymptomatic or has not yet produced a peripheral lesion. This result indicates that at the time of sacrifice, fewer guinea pigs infected with HSV2-LAT-E1-R were experiencing reactivations than HSV-2 or HSV2-LAT-E1, although peripheral lesions were not observed.

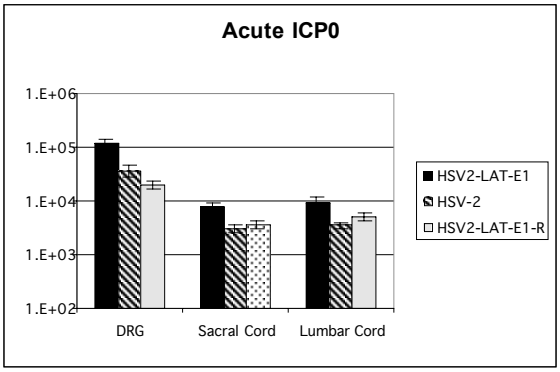
Interestingly, all three of the viruses expressed less LAT in the lumbar spinal cord than in the DRG or sacral cord, although the DNA levels were comparable between the lumbar and sacral regions of the spinal cord. The viruses expressed an average of 14.22 copies of the LAT transcript per viral genome in the DRG (all were similar) and approximately one copy of the LAT transcript per viral genome in the sacral cord (~1.2/genome for HSV-2 and HSV2-LAT-E1-R and 0.7/genome for HSV2-LAT-E1). In the lumbar spinal cord, the viruses averaged approximately 0.1 copies of the LAT transcript per viral genome. Thus, each viral genome expresses approximately 10 times more LAT in the sacral spinal cord and 140 times more LAT in the DRG compared to the lumbar cord. These findings are consistent with the hypothesis that HSV-2 does not efficiently establish true latency in the lumbar cord. Although large quantities of viral DNA are present in the lumbar cord, the failure to express LAT argues against reactivation competence from this region of the nervous system.

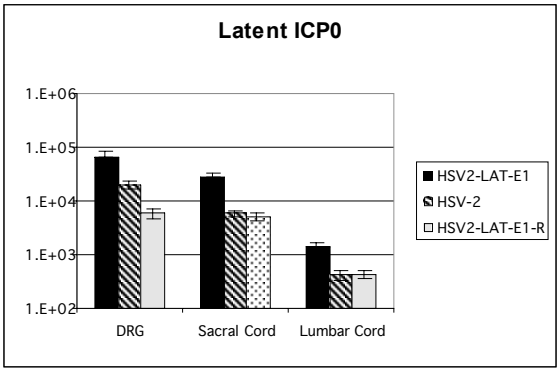
Figure 24. LAT, ICP0, and *tk* Transcript Expression in DRG, Sacral Spinal Cord, and Lumbar Spinal Cord during Acute and Latent Infection.

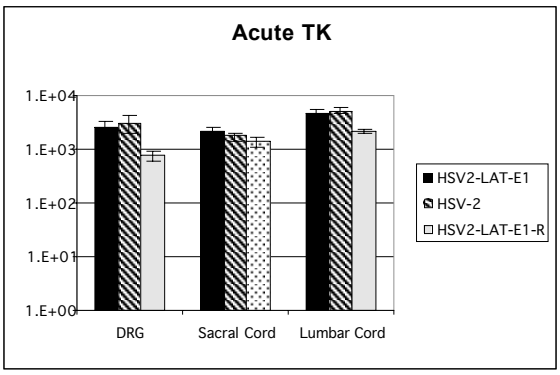
RNA extracted from the lumbosacral DRG, sacral spinal cord, and lumbar spinal cord during acute infection (Day 8 p.i.) and latent infection (Day 42 p.i.), quantified by Taqman PCR assay and normalized to the 18S ribosomal RNA. * $p<0.05$ compared to HSV-2. ** $p<0.05$ compared to rescuant HSV2-LAT-E1-R. Acute: HSV2-LAT-E1 (n=11), HSV-2 (n=10), HSV2-LAT-E1-R (5). Latent: HSV2-LAT-E1 (n=14), HSV-2 (n=9), HSV2-LAT-E1-R (n=8). *LAT exon 1 does not significantly affect the transcription of LAT, ICP0, or tk, therefore changes in viral gene expression do not correlate with differences in reactivation.*

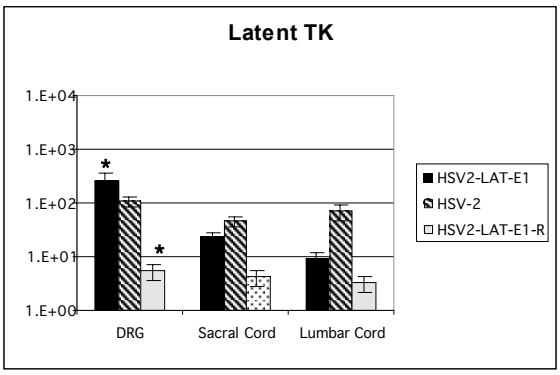

Acute LAT
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Latent LAT
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Acute ICP0
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Latent ICP0
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Acute TK
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R


Latent TK
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
DRG
Sacral Cord
Lumbar Cord
HSV2-LAT-E1
HSV-2
HSV2-LAT-E1-R

Discussion

Previous work with chimeric viruses HSV2-LAT-P1 and HSV2-LAT-S1 demonstrated that the essential elements for the reactivation of HSV-2 resided in the LAT sequence downstream of the promoter rather than within the promoter itself. To further define the region of LAT essential for HSV-2 reactivation, chimeric virus HSV2-LAT-E1 was constructed by replacing the region from the HSV-2 LAT TATA box to the 5' splice site of the LAT intron with the corresponding region from HSV-1. A rescuant was also constructed, restoring the native HSV-2 LAT sequences.

HSV2-LAT-E1 caused an acute infection similar to wild type HSV-2 in lesion severity, but resulted in increased involvement of the autonomic nervous system as shown by increased bladder dysfunction in infected guinea pigs. During latency, HSV2-LAT-E1 reactivated inefficiently in the guinea pig genital model compared to wild type HSV-2 or the rescuant HSV2-LAT-E1-R. Therefore, elements within the LAT exon 1 confer the type-specific recurrence phenotype to HSV-2.

Replacement of HSV-2 LAT exon 1 with HSV-1 LAT exon 1 increased the percentage of guinea pigs experiencing urinary tract dysfunction during the acute phase of infection, indicative of autonomic nervous system involvement, compared to wild type HSV-2 or the rescuant. Increased autonomic nervous system involvement would be expected to coincide with an increased viral load in the sacral or lumbar spinal cord, which contain the parasympathetic and sympathetic nuclei, respectively. However, the mutation did not significantly affect viral load in the sacral or lumbar spinal cord, or in the DRG. Differences in viral load may be found in the major pelvic ganglia, which contain the pelvic post-ganglionic autonomic neurons, although the mechanism for

increased autonomic nervous system involvement cannot be explained based on the tissues evaluated.

HSV2-LAT-E1 did not differ significantly from wild type HSV-2 or the rescuant HSV2-LAT-E1-R within the parameters evaluated. With minor variations, HSV2-LAT-E1 behaved like wild type HSV-2 in its ability to spread through the nervous system and replicate efficiently in the peripheral and central nervous systems. The chimeric virus established latency at levels comparable to HSV–2 and the rescuant. HSV2-LAT-E1 also did not differ significantly from HSV-2 or the rescuant in viral gene expression of the latency-associated transcript, ICP0, or thymidine kinase in the DRG or regions of the spinal cord. However, HSV2-LAT-E1 did not reactivate efficiently after genital inoculation.

In collaboration with Todd Margolis of The Proctor Foundation at University of California San Francisco, HSV2-LAT-E1 was evaluated for neuron-specific establishment of latency in A5+ and KH10+ neurons. HSV-1 preferentially establishes latency in A5+ neurons while HSV-2 preferentially establishes latency in KH10+ neurons. HSV2-LAT-E1 established latency preferentially in A5+ neurons, while the rescuant HSV2-LAT-E1-R preferentially established latency in KH10 + neurons. These results strongly suggest that the LAT exon 1 confers type-specific reactivation by regulating the establishment of latency in specific subtypes of neurons that support latency and reactivation.

Chapter 5

LAT Influences the Differential Spread of HSV-1 and HSV-2 in the Nervous System by Affecting Viral Replication and Lytic Cycle Gene Expression in the DRG and the Spinal Cord

Introduction

HSV-1 and HSV-2 spread preferentially to different regions of the nervous system. HSV-2 spreads more efficiently to the sacral spinal cord, while HSV-1 spreads more efficiently to the lumbar spinal cord. In the spinal cord, LAT appears to influence the efficiency of viral replication differently in the sacral cord and the lumbar cord, as evidenced by viral DNA levels observed with HSV2-LAT-P1 and HSV2-LAT-S1.

To characterize differences between HSV-1 and HSV-2 and also determine the influence of the LAT region on viral spread and the distribution of latency, chimeric virus HSV-2/LAT1 was evaluated and compared to wild type viruses HSV-1 and HSV-2 in the guinea pig footpad model. HSV-2/LAT1 is HSV-2 in which the LAT region has been replaced by the corresponding region of HSV-1, including the promoter, exon 1, and most of the LAT intron. I developed the footpad model to evaluate viral spread clinically, rather than by post-mortem tissue examination at various time points post-inoculation, to limit the number of guinea pigs that must be sacrificed during the course of disease including acute and latent infection. The footpad model was employed to characterize differences in the ability of HSV-1 and HSV-2 to spread to the central nervous system from a peripheral site of inoculation that is innervated primarily by sensory neurons without the extensive autonomic nervous system involvement observed

in the genital model. By inoculating in the footpad, the virus has limited access to sympathetic and parasympathetic nerve fibers. Although some autonomic nerve fibers do reach the footpad, primarily sympathetic fibers surrounding blood vessels, the density of autonomic innervation is much less than that found in the cervicovaginal epithelium. Therefore, the virus is directed through primarily the sensory pathway to the spinal cord. Inoculation into a single footpad provides a model by which viral spread can be observed through the sensory pathway into and through the central nervous system to the contralateral DRG, which could provide insight into the differences between HSV-1 and HSV-2 in their abilities to spread through multiple transneuronal synapses. The footpad model also provides a means of observing differences in the specific pathways that the viruses choose to follow by evaluating differences in viral load in different regions of the nervous system. Expression of LAT, ICP0, and thymidine kinase transcripts were also evaluated and compared in the footpad model.

To determine the role of the LAT region in the expression of viral genes associated with latency and reactivation, transcript expression of LAT, ICP0, and thymidine kinase was also evaluated after genital inoculation of wild type HSV-2, HSV-2/LAT1 and a LAT promoter-deleted HSV-2 mutant, ΔLAT. ΔLAT does not express LAT during latency and provides information regarding the role of the HSV-2 LAT promoter. By expressing the HSV-1 LAT region in place of the HSV-2 LAT region, HSV-2/LAT1 allows for comparisons between the HSV-1 and HSV-2 LAT regions and their influence on pathogenesis, replication and viral gene expression. The genital model was evaluated in addition to the footpad to gain insight into the relative influence of the autonomic nervous system. The cervicovaginal epithelium in densely innervated by both

sympathetic and parasympathetic nerve endings in addition to sensory nerve endings and the viruses appear to utilize the autonomic nervous system pathways differently. By comparing the footpad and the genital models, we can determine if the autonomic nervous system plays a definitive role in viral spread into and through the nervous system.

Results

HSV-1, HSV-2, and chimeric HSV2/LAT1 have similar acute lesion severity after footpad inoculation.

To characterize differences between HSV-1 and HSV-2 spread and the effect of LAT on viral spread, guinea pigs were inoculated by unilateral injection into the right hind footpad with 2 x 10^5 pfu of wild type viruses HSV-2 and HSV-1 as well as the chimeric virus HSV2/LAT1. The guinea pigs were evaluated for lesion severity during the acute disease for 14 days post-inoculation. There were no significant differences in acute lesion severity between the HSV-2 and HSV-1 ($p=0.057$) or HSV-2 and HSV-2/LAT-1 ($p=0.598$) after footpad inoculation, although the overall severity of the acute infection was less than typically observed in the guinea pig genital model (Figure 25). All acute lesions occurred only on the inoculated footpad in infected guinea pigs.

LAT confers type-specific reactivation after footpad inoculation.

Consistent with the guinea pig genital model of HSV infection, HSV-2 reactivated efficiently while HSV-1 was impaired for reactivation after footpad inoculation (Figure 26). By expressing HSV-1 LAT sequences in the context of HSV-2, HSV2/LAT1 was

also impaired for reactivation, with a recurrence frequency more similar to HSV-1 ($p=0.114$) than HSV-2 ($p=0.001$). This result is also consistent with the guinea pig genital model of infection, demonstrating that the LAT confers the type-specific recurrence phenotype regardless of route of inoculation. Recurrent lesions were observed on the ipsilateral footpad and the external genitalia in guinea pigs infected with HSV-2 or HSV-1 (Table 2). Approximately the same number of recurrent lesions was observed on the ipsilateral footpad and the external genitalia during the HSV-2 latent infection (35 and 30 of 66 total recurrent lesions, respectively), and a single lesion was observed on the contralateral footpad in one guinea pig infected with HSV-2. Although guinea pigs infected with HSV-1 had few recurrent lesions, 75% of the recurrent lesions (6 of 8) were observed on the external genitalia while 25% (2 of 8) appeared on the ipsilateral footpad. In animals infected with HSV-2/LAT1, 5 of the 30 total recurrent lesions (16.67%) were observed on the contralateral footpad in addition to those occurring on the ipsilateral footpad (8 of 30 total recurrent lesions) and external genitalia (17 of 30). These clinical results suggest that by expressing HSV-1 LAT in the context of an HSV-2 virus, HSV-2/LAT1 had an increased ability to spread through the nervous system to reach the contralateral DRG. The multiple recurrent lesions observed on the contralateral footpad during HSV-2/LAT1 latent infection provide evidence that the chimeric virus spread through the central nervous system and established reactivation-competent latency in the contralateral DRG. Only 1.52% of HSV-2 recurrent lesions occurred on the contralateral footpad and 16.67% of HSV-2/LAT1 recurrent lesions occurred on the contralateral footpad, suggesting that one function of the native HSV-2 LAT region is to limit spread of the virus.

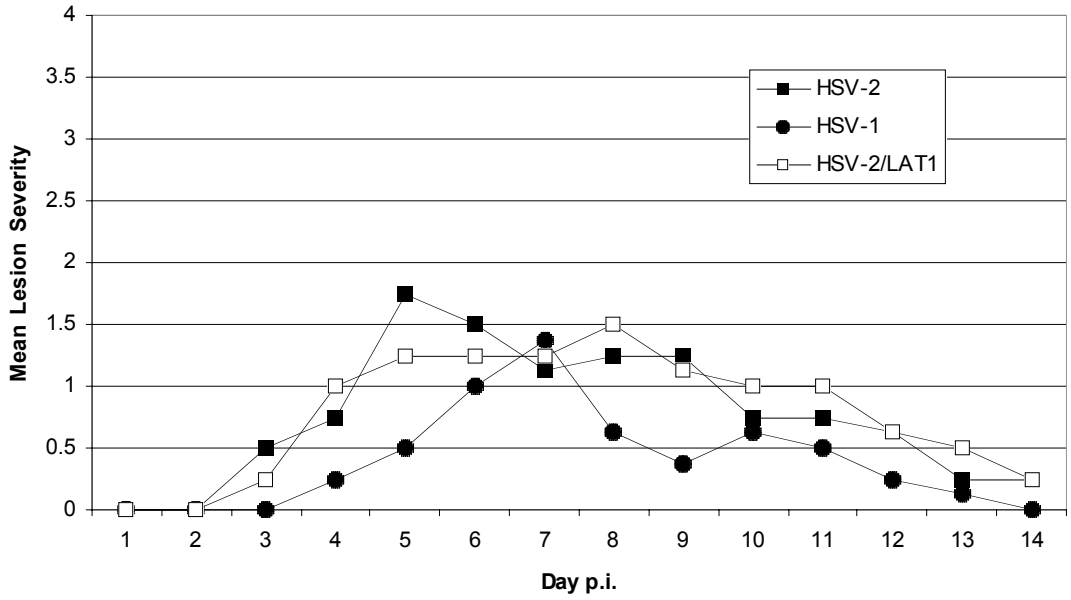


Figure 25. Acute Lesion Severity after Footpad Inoculation - HSV-2, HSV-1, HSV-2/LAT1.

Lesion severity is graphed as the mean for each group of guinea pigs on each day of observation from Days 1-14 p.i., with 0 being no symptoms and 4 being the most severe. HSV-2 (n=8), HSV-1 (n=8), HSV-2/LAT1 (n=8). All acute lesions occurred on the inoculated footpad. *Acute lesion severity was similar between the viruses after footpad inoculation, suggesting that LAT does not affect acute lesion severity after footpad inoculation.*

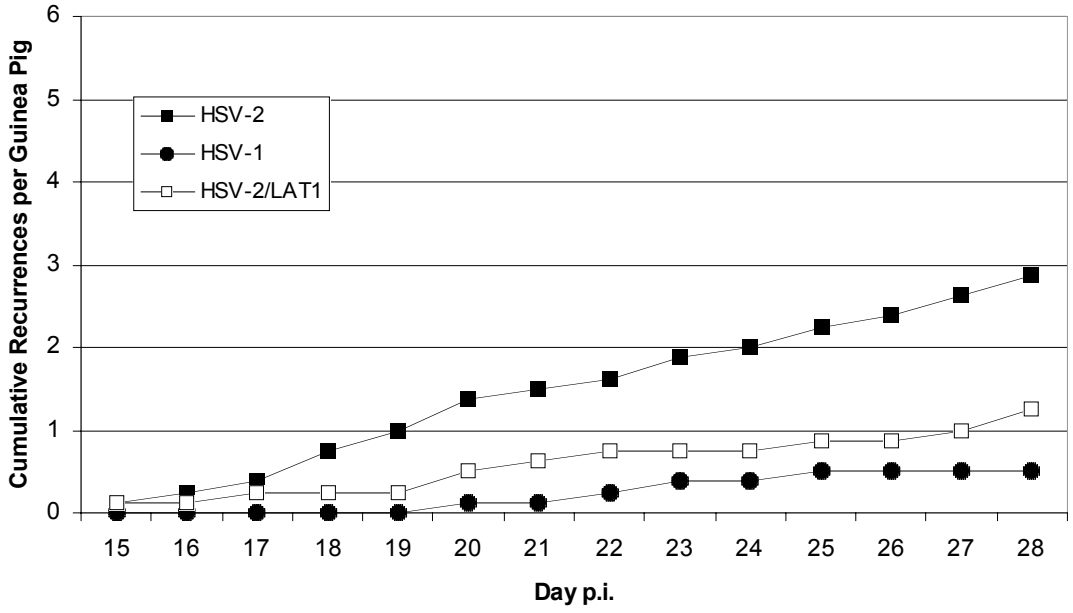


Figure 26. Recurrences after Footpad Inoculation - HSV-2, HSV-1, HSV-2/LAT1.

Cumulative recurrences per guinea pig for each group during latent infection. HSV-2 (n=8), HSV-1 (n=8), HSV-2/LAT1 (n=8). Mann-Whitney p values are as follows: HSV-2 vs. HSV-1 = 0.001, HSV-2 vs. HSV-2/LAT1 = 0.001, HSV-1 vs. HSV-2/LAT1 = 0.114. *HSV-2 reactivated more efficiently than HSV-1 after footpad inoculation, and the reduced reactivation frequency by HSV-2/LAT1 demonstrates that the LAT region confers the type-specific reactivation phenotype.*

	Right Foot Lesions	Genital Lesions	Left Foot Lesions
HSV-2	**53.03%** 35/66	**45.45%** 30/66	**1.52%** 1/66
HSV-1	**25.00%** 2/8*	**75.00%** 6/8*	**0.00%** 0/8
HSV-2/LAT1	**26.67%** 8/30*	**56.67%** 17/30	**16.67%** 5/30*[#]

Table 2. Anatomical Site of Recurrences after Footpad Inoculation.

The anatomical sites of the recurrences were documented for each guinea pig. Recurrences are presented as a percentage of total recurrences occurring on the right footpad, left footpad, or external genitalia (Genital Lesions). The bottom numbers represent cumulative number of recurrent lesions observed at that site/total number of recurrent lesions observed for each group. * $p<0.005$ compared to HSV-2. [#] $p=0.027$ compared to HSV-1. *Contralateral recurrences after HSV-2/LAT1 infection suggest that the HSV-1 sequences in the context of HSV-2 permitted increased spread through the CNS into the contralateral DRG.*

HSV-2 and HSV-1 spread preferentially to different regions of the CNS, influenced by LAT.

To determine the effects of LAT on latent viral load and the biodistribution of latent infection, infected guinea pigs were sacrificed 90 days post-inoculation and nervous system tissues were evaluated for latent viral load by Taqman quantitative real-time PCR assay. After inoculation into the right hind footpad, HSV-2 spread more efficiently to the sacral spinal cord than the lumbar cord (Figure 27). HSV-1, however, spread efficiently to the lumbar spinal cord but viral DNA was undetectable in the sacral spinal cord. Viral DNA levels of HSV-1 were 1.5 logs higher in the lumbar cord than in the DRG. Neither HSV-2 nor HSV-1 DNA was detected in the left DRG, suggesting that the wild type viruses were unable to spread efficiently through the spinal cord to reach and replicate in the contralateral DRG.

HSV-2/LAT1 DNA was detected in the right DRG at levels approximately 20 times higher than HSV-2, suggesting that expression of the HSV-1 LAT sequence permitted increased viral replication within the right DRG. HSV-2/LAT1 spread efficiently to both the sacral and lumbar regions of the spinal cord, with DNA levels in the sacral spinal cord similar to HSV-2 sacral DNA levels ($p=0.681$) and in the lumbar cord similar to HSV-1 lumbar DNA levels ($p=0.118$). Although neither HSV-2 nor HSV-1 DNA was detectable in the contralateral DRG, HSV-2/LAT1 viral DNA was detected in the left DRG of 5 of the 8 infected animals ($p=0.008$ compared to HSV-2 and 0.013 compared to HSV-1). Therefore, by expressing HSV-1 LAT, HSV-2 gained the ability to replicate more efficiently in the ipsilateral DRG and spread more efficiently through the central nervous system to the contralateral DRG.

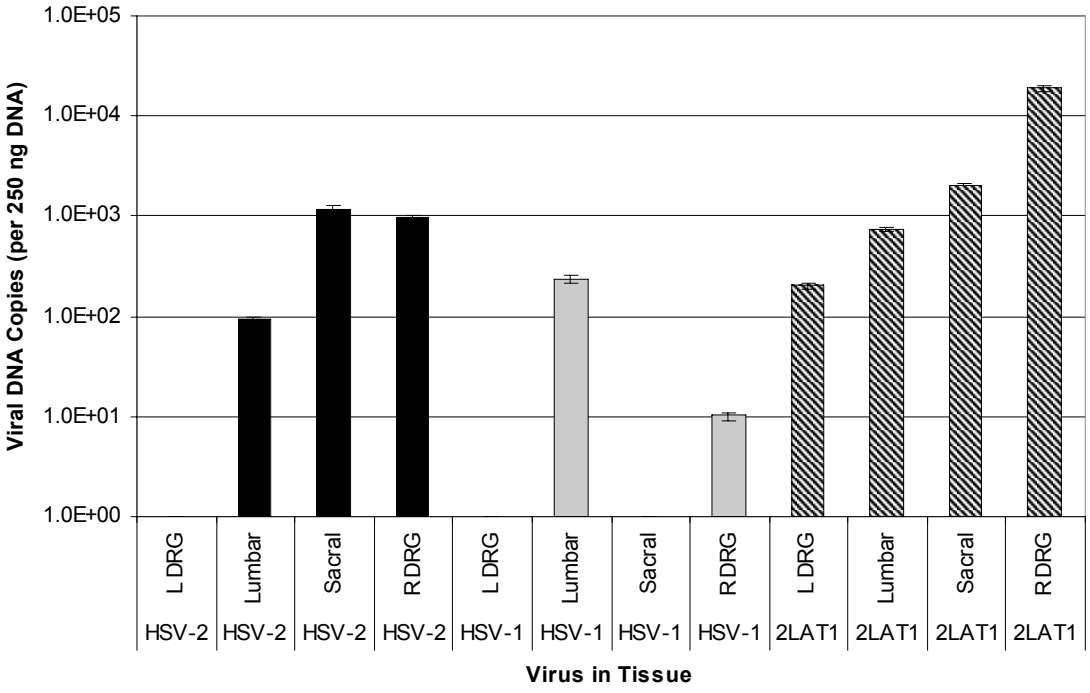


Figure 27. Tissue Distribution of Latent Viral DNA after Footpad Inoculation.

Viral DNA extracted from the lumbosacral DRG, sacral spinal cord, and lumbar spinal cord was quantified by Taqman PCR assay and normalized to the gene for the 18S ribosomal RNA. HSV-2 vs. HSV-1: R DRG p=0.008, Sacral p=0.016. HSV-2 vs. HSV-2/LAT1: L DRG p=0.017. HSV-1 vs. HSV-2/LAT1: R DRG p=0.008, Sacral p=0.013, L DRG p=0.017. *By expressing HSV-1 LAT, HSV-2/LAT1 gained the ability to replicate more efficiently in the ipsilateral DRG and spread more efficiently through the central nervous system to the contralateral DRG.*

Type-specific LAT influences viral gene expression differently in DRG and spinal cord regions.

RNA from tissues from the guinea pigs infected with HSV-2/LAT1 via the footpad was also evaluated to determine the effect of LAT on viral transcript expression during latency. Thymidine kinase (*tk*) transcript levels were undetectable in all tissues evaluated, indicating latency in all animals since *tk* is expressed only during replication.

Although DNA was undetectable in the left DRG for both HSV-1 and HSV-2, LAT expression was readily detected, indicating that small quantities of HSV-1 and HSV-2 must have reached the left DRG but were below the limits of detection in our assays (<10 copies of viral DNA in 50 ng of total DNA) (Figure 28). LAT expression was also detected in the sacral spinal cord in animals infected with HSV-1 at levels similar to the lumbar spinal cord in animals infected with HSV-1, although viral DNA was below the level of detection in the sacral cord. In animals infected with HSV-2/LAT1, LAT expression in the left DRG was comparable to LAT expression in the sacral spinal cord, although the left DRG had 10-fold less DNA than the sacral cord.

ICP0 transcript expression was also detectable in the left DRG in guinea pigs infected with HSV-2, although the DNA was undetectable (Figure 29). The quantity of ICP0 transcript detected was similar to that detected in the right DRG, although the right DRG contained at least 100-fold more DNA than the left DRG, given the limits of detection for our assays. HSV-1 ICP0 transcript expression was not detected in the left DRG but was apparent in the sacral spinal cord, although viral DNA was below the limits of detection in both of these tissue sites. In guinea pigs infected with HSV-2/LAT1,

ICP0 transcript was expressed in the left DRG at levels comparable to that expressed in the sacral cord, although the left DRG harbored 10-fold less DNA than the sacral cord.

LAT expression differed according to tissue type (Table 3). Each DNA copy of HSV-2 expressed greater quantities of LAT in the DRG (~26 copies of LAT/DNA copy) and sacral cord (~10 copies of LAT/DNA copy) than in the lumbar cord (0.66 copies of LAT/DNA copy). ICP0 expression from HSV-2 was similar among the tissue types (0.09-0.12 copies of ICP0/DNA copy). HSV2/LAT1 expressed comparatively higher levels of LAT and ICP0 than HSV-2 in the DRG and spinal cord. In the right DRG, each copy of HSV-2/LAT1 expressed ~68 copies of LAT and 0.30 copies of ICP0 compared to ~26 copies of LAT and 0.12 copies of ICP0 per HSV-2 DNA. In the sacral cord, each copy of HSV2/LAT1 expressed 155 copies of LAT and 0.54 copies of ICP0 while HSV-2 expressed only ~10 copies of LAT and 0.09 copies of ICP0 per genome. These results suggest that the type-specific LAT region regulates expression of LAT and ICP0 differently in different tissue types.

In the left DRG, each DNA copy of HSV2/LAT1 produced ~869 copies of LAT transcript compared to ~155 LAT transcripts per DNA copy in the sacral cord. The LAT expression per DNA copy from HSV2/LAT1 was also more than 10-fold higher in the left DRG (~869 LAT copies/DNA) than in the right DRG (~68 LAT copies/DNA). HSV-2/LAT1 also expressed nearly 10-fold higher quantities of ICP0 transcript per viral genome in the left DRG compared to the right DRG. These results suggest differences in LAT and ICP0 expression depending on whether the virus reached the DRG from a peripheral site or from the central nervous system.

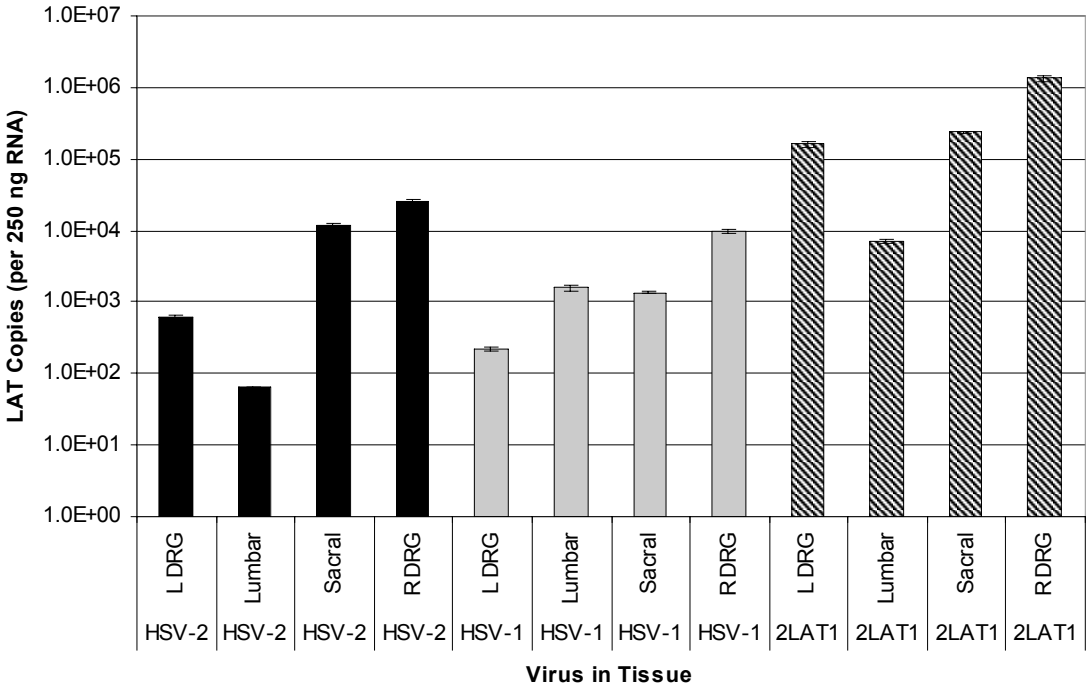


Figure 28. LAT Expression after Footpad Inoculation.

RNA extracted from right DRG, sacral spinal cord, lumbar spinal cord, and left DRG, quantified for LAT transcript expression by Taqman RT-PCR assay, and normalized to the 18S ribosomal RNA. In the R DRG, HSV-2/LAT1 was statistically significant from HSV-2 ($p=0.029$) and HSV-1 ($p=0.021$). *LAT transcript was expressed in left DRG although DNA was below limits of detection, suggesting that small quantities of HSV-2 and HSV-1 did spread to the contralateral DRG, although those small quantities in the contralateral DRG expressed greater quantities of LAT transcript per viral genome than the virus present in the ipsilateral DRG.*

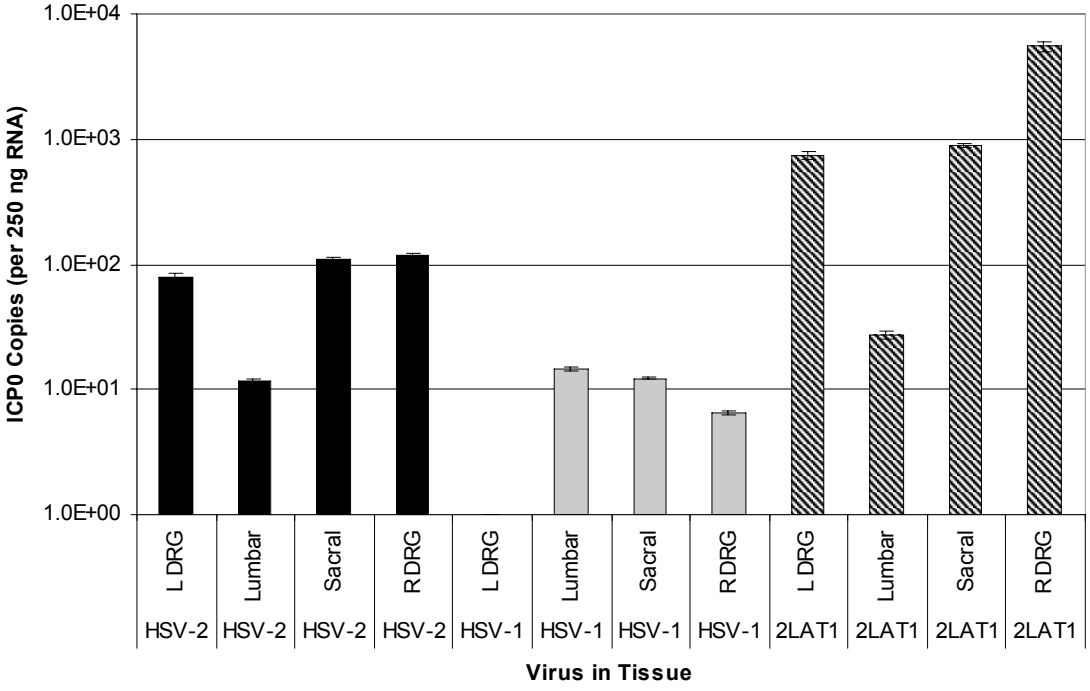


Figure 29. ICP0 Expression after Footpad Inoculation.

RNA extracted from right DRG, sacral spinal cord, lumbar spinal cord, and left DRG, quantified for ICP0 transcript expression by Taqman RT-PCR assay, and normalized to the 18S ribosomal RNA. HSV-2 vs. HSV-1: R DRG p=0.004, L DRG p=0.005. HSV-2 vs. HSV-2/LAT1: Sacral p=0.031. HSV-1 vs. HSV-2/LAT1: R DRG p=0.007, Sacral p<0.0005, L DRG p=0.012. *ICP0 was expressed in left DRG after HSV-2 but not HSV-1 infection although DNA was below limits of detection, suggesting that HSV-2 is latent and reactivation competent while HSV-1 is latent but not reactivation competent in the contralateral DRG after viral spread from the spinal cord.*

	LAT Transcripts/Viral Genome			ICP0 Transcripts/Viral Genome		
	HSV-2	**HSV-1**	**HSV-2/LAT1**	**HSV-2**	**HSV-1**	**HSV-2/LAT1**
R DRG	25.9	110.09	68.15	0.12	0.25	0.30
Sacral	9.69	*	155.27	0.09	*	0.54
Lumbar	0.66	6.36	10.18	0.11	0.03	0.04
L DRG	*	*	868.87	*	*	2.36

* No detectable DNA

Table 3. LAT and ICP0 Expression Comparison after Footpad Inoculation.

In guinea pigs with detectable DNA, the quantities of LAT and ICP0 transcripts were divided by the copies of viral DNA to obtain the ratio of the number of transcripts expressed per viral genome. The data in the table represent the mean number of LAT or ICP0 transcripts expressed per viral genome for each group of guinea pigs. *The type-specific LAT region regulates expression of LAT and ICP0 differently in different tissue types. More LAT/genome is expressed after viral spread from the spinal cord compared to viral spread from the periphery, suggesting differences in establishment of latency between virus entering the DRG from the CNS and from the periphery.*

Type-specific LAT limits acute lesion severity in the genital model of infection.

To determine if HSV-2 LAT influences viral spread and the establishment of latency through an effect on viral gene expression, guinea pigs were inoculated intravaginally with 2 x 10^5 pfu of HSV-2, HSV2/LAT1 and ΔLAT, a LAT transcription-negative mutant of HSV-2. The mean acute lesion scores for HSV2/LAT1 and ΔLAT were both greater than wild type HSV-2, suggesting that elements within the HSV-2 LAT region play a role in limiting the severity of the acute genital infection caused by HSV-2 (Figure 30).

LAT confers type-specific reactivation after genital inoculation.

After genital inoculation, the LAT mutants reactivated less efficiently than WT HSV-2 (Figure 31). By expressing HSV-1 LAT within the context of the HSV-2 genome, the chimeric virus demonstrated a 60% reduction in its ability to reactivate compared to wild type HSV-2 ($p=0.034$), although it reactivated more efficiently than the LAT-negative mutant ($p=0.022$ compared to HSV-2). These results demonstrate that the LAT region confers type-specific reactivation competence to HSV-2. HSV-1 LAT expressed in the context of the HSV-2 genome permits limited reactivation, but HSV-2 LAT sequences are required for the characteristic genital HSV-2 recurrence phenotype.

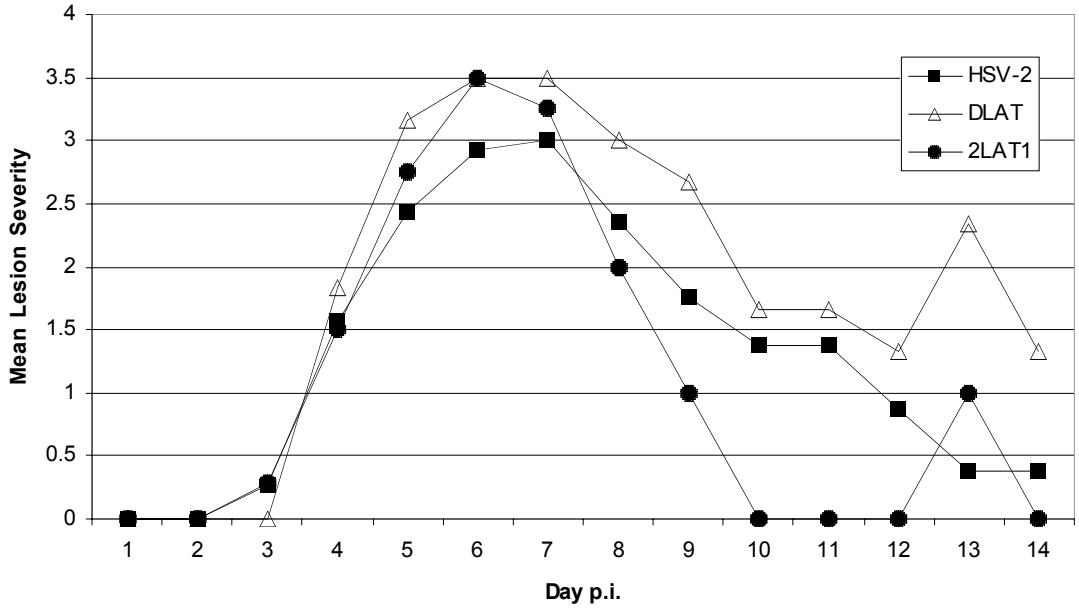


Figure 30. Acute Lesion Severity after Genital Inoculation - HSV-2, ΔLAT and HSV-2/LAT1.

Lesion severity graphed as the mean for each group of guinea pigs on each day of observation from Days 1-14 p.i., on a scale from 0 to 4, with 0 being no symptoms and 4 being the most severe. HSV-2 (n=14), ΔLAT (n=6), HSV-2/LAT1 (n=5). *The mean acute lesion scores for HSV2/LAT1 and ΔLAT were both greater than wild type HSV-2, suggesting that elements within the HSV-2 LAT region play a role in limiting the severity of the acute genital infection caused by HSV-2***.**

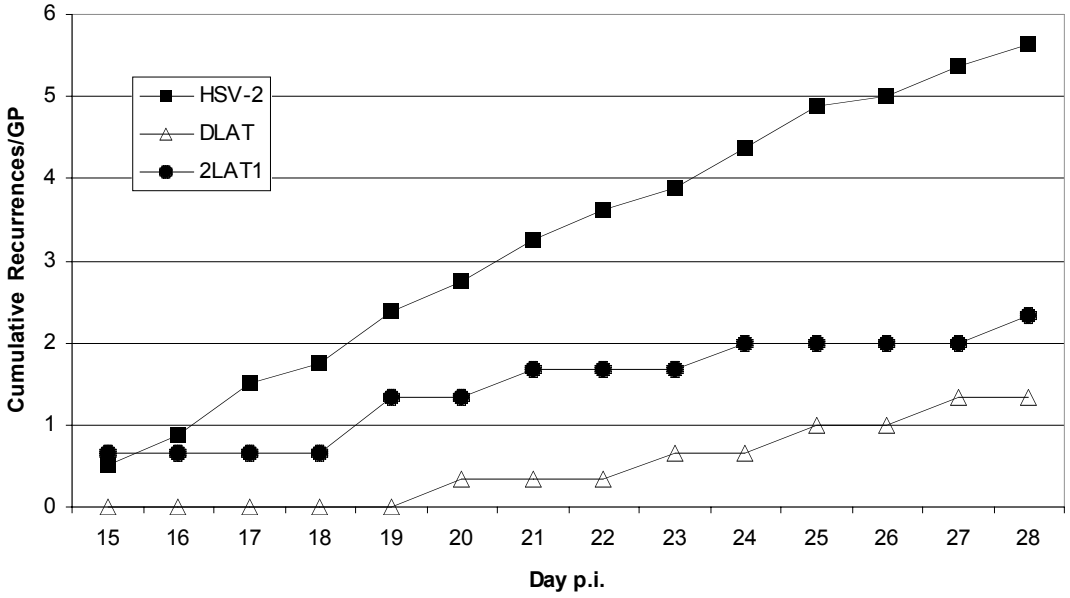


Figure 31. Cumulative Recurrences after Genital Inoculation - HSV-2, HSV-1, ΔLAT and HSV-2/LAT1.

Cumulative recurrences per guinea pig in each group. HSV-2 (n=8), HSV-1 (n=4), ΔLAT (n=3), HSV-2/LAT1 (n=3). HSV-2 vs. ΔLAT (p=0.022) and HSV-2 vs. HSV-2/LAT1 (n=0.034). *HSV-2/LAT1 and ΔLAT reactivate less efficiently than HSV-2, demonstrating that the LAT region confers type-specific reactivation.*

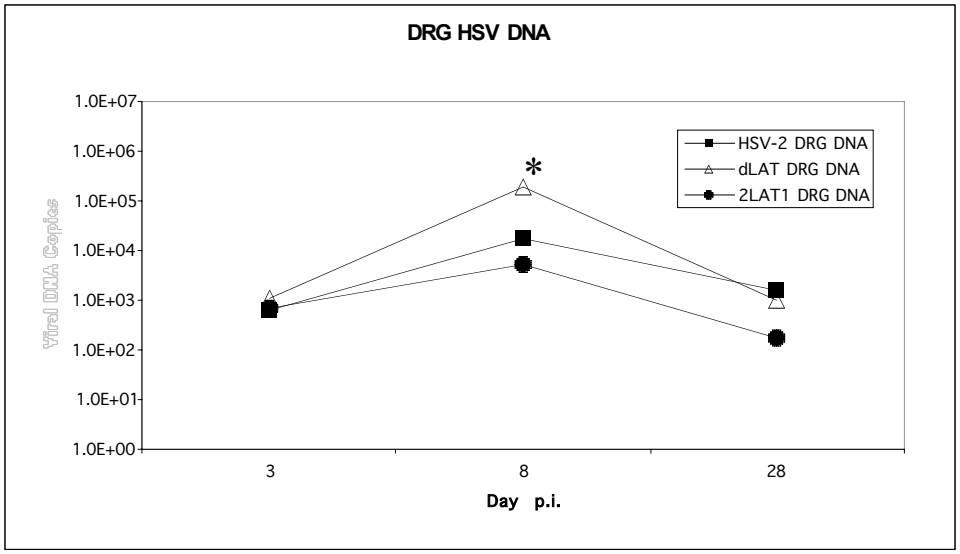
LAT influences viral DNA levels in DRG and sacral spinal cord after genital infection.

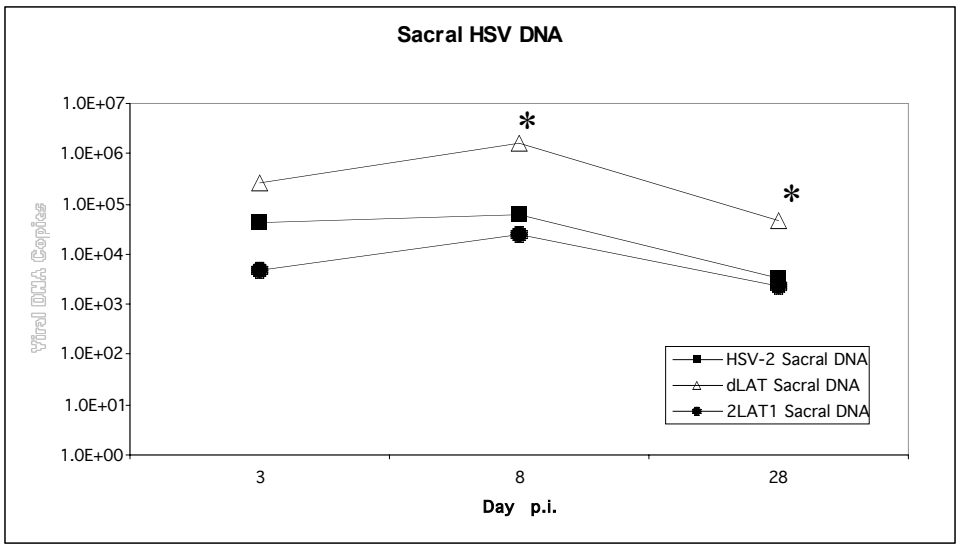
To determine the effects of the HSV-2 LAT region on viral replication, intravaginally-infected guinea pigs were sacrificed at time points during early infection prior to the appearance of symptoms (Day 3), peak acute disease (Day 8), and latency (Day 28). At each time point, viral DNA levels were evaluated by Taqman quantitative PCR assay.

In the DRG, ΔLAT demonstrated a significantly higher level of viral DNA on Day 8 at the peak of acute infection ($p=0.026$), although the latent viral DNA levels were similar (Figure 32). In the sacral spinal cord, ΔLAT demonstrated increased levels of viral DNA throughout the acute infection and during latency (<0.001). No differences were observed in the lumbar cord. These results demonstrate that without LAT, HSV-2 is able to replicate more efficiently in the DRG and the sacral spinal cord and is capable of establishing latency comparable to wild type HSV-2, although it is unable to efficiently reactivate. Viral DNA levels of HSV-2/LAT1 were similar to wild type HSV-2 in the DRG and the spinal cord, implying that some LAT elements contributing to efficient replication are present in both HSV-2 and HSV-1 LAT regions. However, the elements contributing to efficient viral replication do not appear to contribute to type-specific reactivation, as HSV-2/LAT1 fails to reactivate efficiently even with viral DNA levels comparable to wild type HSV-2.

Figure 32. Viral DNA in DRG, Sacral Cord, and Lumbar Cord after Genital Inoculation.

DNA was extracted from DRG, sacral spinal cord, and lumbar spinal cord. Viral DNA was quantified by Taqman real-time PCR assay and normalized to the gene for the 18S ribosomal RNA. Viral DNA quantities were log-transformed for statistical analysis. * $p<0.03$ compared to HSV-2 at the same time point. HSV-2 (n=6 at each time point), ΔLAT (n=3 at each time point), HSV-2/LAT1 (n=3 at each time point). *ΔLAT demonstrates that without LAT, HSV-2 is able to replicate more efficiently in the DRG and the sacral spinal cord and is capable of establishing latency comparable to wild type HSV-2, although it is unable to efficiently reactivate. HSV-2/LAT1 demonstrates that some LAT elements contributing to efficient replication are present in both HSV-2 and HSV-1 LAT regions, although the elements contributing to efficient viral replication do not appear to contribute to type-specific reactivation, as HSV2/LAT1 fails to reactivate efficiently even with viral DNA levels comparable to wild type HSV-2.*


DRG HSV DNA
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Viral DNA Copies
HSV-2 DRG DNA
dLAT DRG DNA
2LAT1 DRG DNA
*
3
8
28
Day p.i.


Sacral HSV DNA
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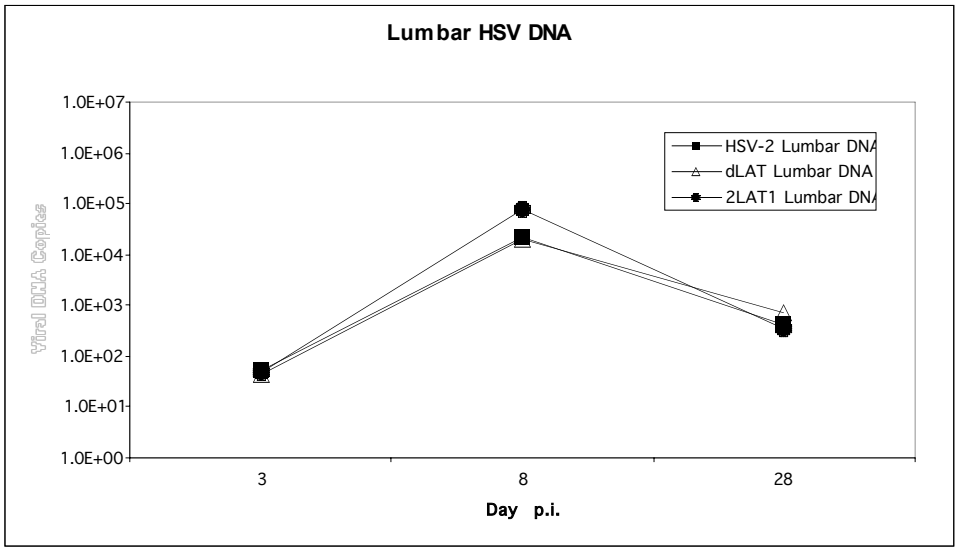
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Viral DNA Copies
HSV-2 Sacral DNA
dLAT Sacral DNA
2LAT1 Sacral DNA
*
*
3
8
28
Day p.i.


Lumbar HSV DNA
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Viral DNA Copies
HSV-2 Lumbar DNA
dLAT Lumbar DNA
2LAT1 Lumbar DNA
3
8
28
Day p.i.

Type-specific LAT influences viral gene expression differently in DRG and spinal cord regions.

To determine the effects of the HSV-2 LAT region on viral gene expression, nervous system tissues were evaluated for transcript expression of LAT, ICP0 (a viral transactivator essential for reactivation) and thymidine kinase (*tk*) by Taqman quantitative RT-PCR.

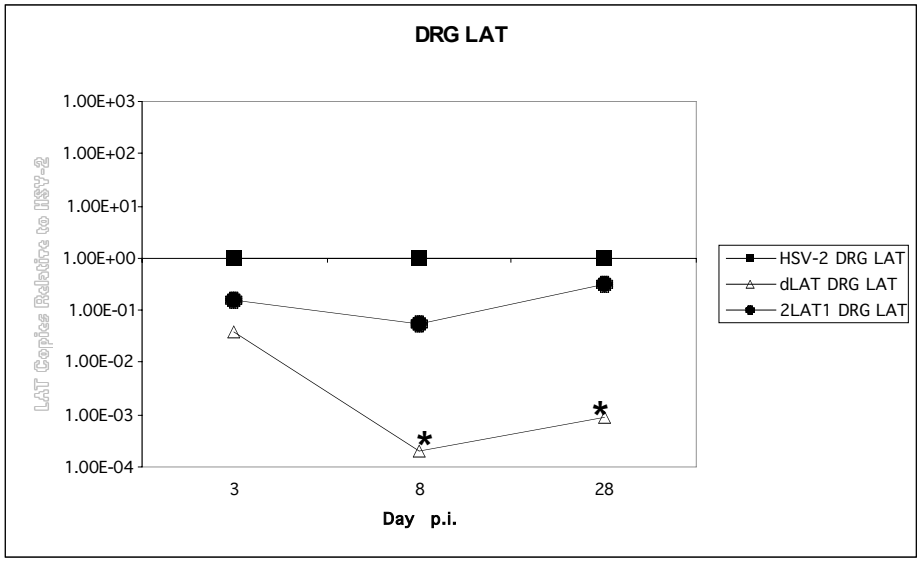
Without the LAT promoter, LAT expression was significantly reduced compared to wild type HSV-2 in all tissues evaluated ($p \leq 0.038$) as expected, except during the acute infection in the lumbar cord where wild type HSV-2 expressed lower levels of LAT than in the DRG and sacral cord (Figure 33). With HSV-2/LAT1, expression of HSV-1 LAT in the context of HSV-2 reduced the levels of LAT expression in the DRG during acute infection but not down to the levels of ΔLAT and differences were not statistically significant compared to HSV-2. In the spinal cord, HSV2/LAT1 expressed significantly greater levels of LAT during acute infection compared with wild type HSV-2 ($p=0.001$ in sacral cord and 0.002 in lumbar cord). These data suggest that HSV-1 and HSV-2 LAT sequences regulate transcription of LAT differently in the DRG and the spinal cord.

In the DRG, ΔLAT and HSV2/LAT1 expressed significantly less ICP0 than HSV-2 during acute infection ($p \leq 0.031$), and reduced levels (although not significantly) during latency (Figure 34). In the spinal cord however, HSV2/LAT1 expressed ICP0 transcript at or above wild type HSV-2 levels while ΔLAT expressed significantly less ICP0 transcript during latency ($p \leq 0.003$), suggesting that the limited recurrences observed during HSV2/LAT1 latency may have arisen from the spinal cord rather than the DRG.

Thymidine kinase (*tk*) is an indicator of active viral replication. In the DRG, ΔLAT expressed wild type HSV-2 levels of *tk* during acute infection, after which expression ceased completely with ΔLAT but was still detectable in wild type HSV-2-infected DRG (p=0.006) (Figure 35). HSV2/LAT1 expressed significantly reduced levels of *tk* during acute infection compared to HSV-2 (p=0.015) and expression during latent infection was undetectable in the DRG (p=0.006 compared to HSV-2). These data suggest that only wild type HSV-2 was capable of replication in the DRG during latency; thus type-specific LAT regions are required for replication in the DRG during latency. In the sacral cord, ΔLAT had significantly decreased levels of *tk* expression compared to HSV-2 (p=0.023) while HSV2/LAT1 had wild type levels of *tk* expression. These data support the hypothesis that reactivation observed during HSV2/LAT1 latency arose from the sacral spinal cord rather than the DRG. There were no significant differences between the viruses in the lumbar cord.

Figure 33. LAT Expression after Genital Inoculation.

RNA was extracted from DRG, sacral spinal cord, and lumbar spinal cord from intravaginally-infected guinea pigs sacrificed prior to the appearance of symptoms (Day 3), during acute infection (Day 8) and during latent infection (Day 28). RNA was normalized to the 18S ribosomal RNA. DNA quantities of ΔLAT and HSV-2/LAT1 were normalized to HSV-2 at each given time point and the RNA quantities were adjusted by the same factor. The quantities presented represent LAT expression relative to the LAT expression of HSV-2 at each given time point. * $p \leq 0.038$ compared to HSV-2 at the same time point. HSV-2 (n=6 at each time point), ΔLAT (n=3 at each time point), HSV-2/LAT1 (n=3 at each time point). *HSV-1 and HSV-2 LAT sequences regulate transcription of LAT differently in the DRG and the spinal cord, suggesting that the LAT regions respond differently to cellular factors within the neurons to regulate LAT transcription.*


DRG LAT
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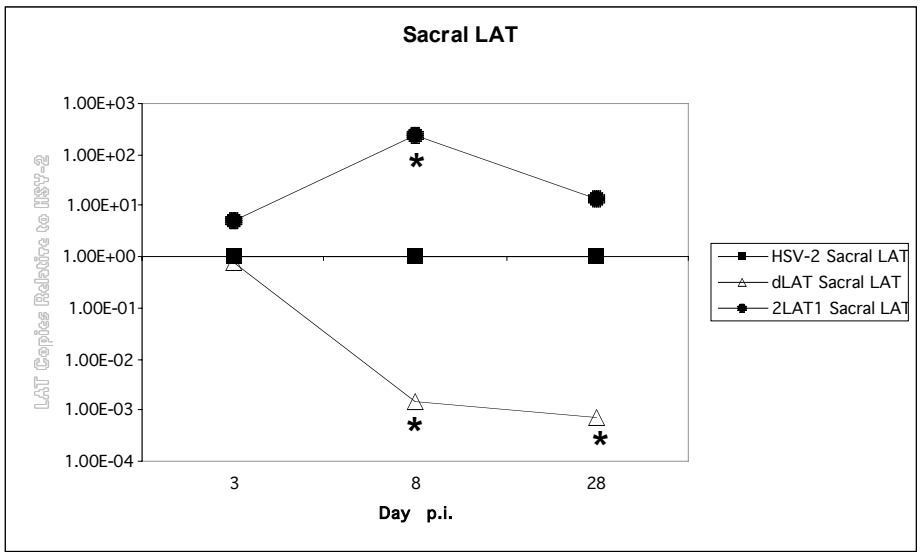
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
LAT Copies Relative to HSV-2
3
8
28
Day p.i.
HSV-2 DRG LAT
dLAT DRG LAT
2LAT1 DRG LAT
*
*


Sacral LAT
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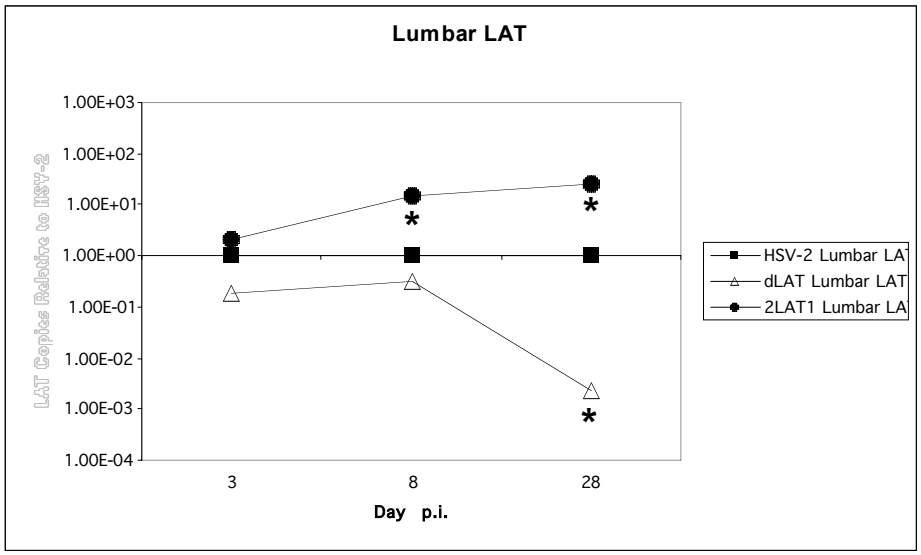
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
LAT Copies Relative to HSV-2
3
8
28
Day p.i.
HSV-2 Sacral LAT
dLAT Sacral LAT
2LAT1 Sacral LAT
*
*
*


Lumbar LAT
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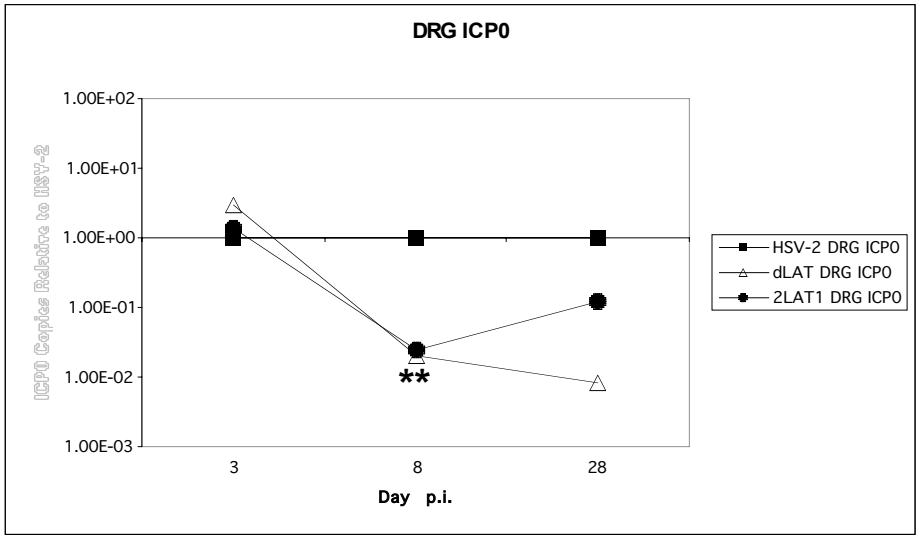
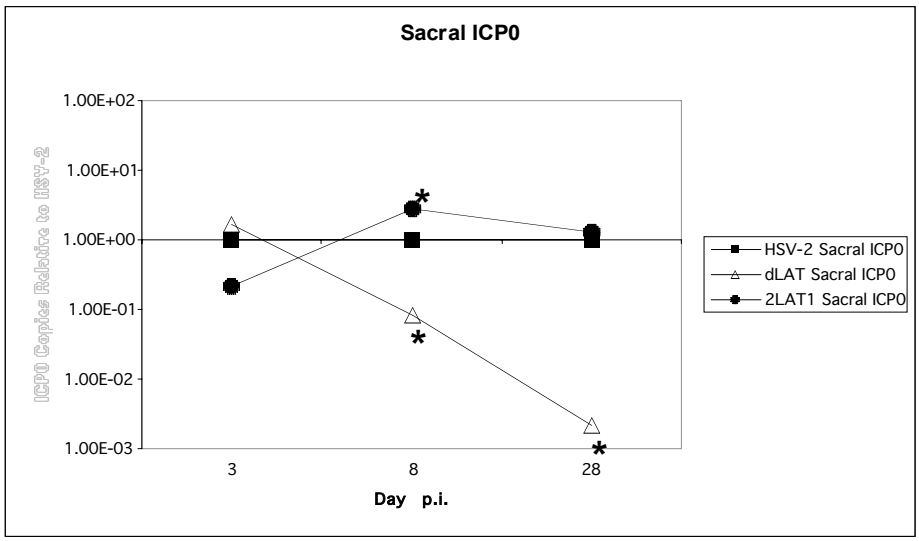
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
LAT Copies Relative to HSV-2
3
8
28
Day p.i.
HSV-2 Lumbar LA
dLAT Lumbar LAT
2LAT1 Lumbar LA
*
*
*

Figure 34. ICP0 Expression after Genital Inoculation.

RNA was extracted from DRG, sacral spinal cord, and lumbar spinal cord from intravaginally-infected guinea pigs sacrificed prior to the appearance of symptoms (Day 3), during acute infection (Day 8) and during latent infection (Day 28). RNA was normalized to the 18S ribosomal RNA. DNA quantities of ΔLAT and HSV-2/LAT1 were normalized to HSV-2 at each given time point and the RNA quantities were adjusted by the same factor. The quantities presented represent LAT expression relative to the LAT expression of HSV-2 at each given time point. * $p \leq 0.046$ compared to HSV-2 at the same time point. HSV-2 (n=6 at each time point), ΔLAT (n=3 at each time point), HSV-2/LAT1 (n=3 at each time point). *Reduced ICP0 in the DRG and wild type levels of ICP0 in the spinal cord suggest that the limited recurrences observed during HSV2/LAT1 latency may have arisen from the spinal cord rather than the DRG.*


DRG ICP0
ICP0 Copies Relative to HSV-2
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
3
8
28
Day p.i.
**
HSV-2 DRG ICP0
dLAT DRG ICP0
2LAT1 DRG ICP0


Sacral ICP0
ICP0 Copies Relative to HSV-2
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
3
8
28
Day p.i.
*
*
*
HSV-2 Sacral ICP0
dLAT Sacral ICP0
2LAT1 Sacral ICP0

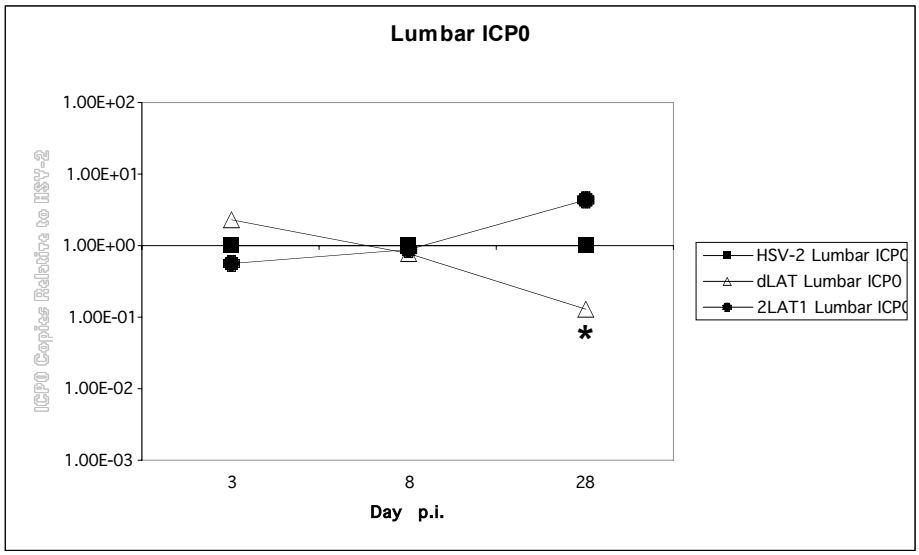
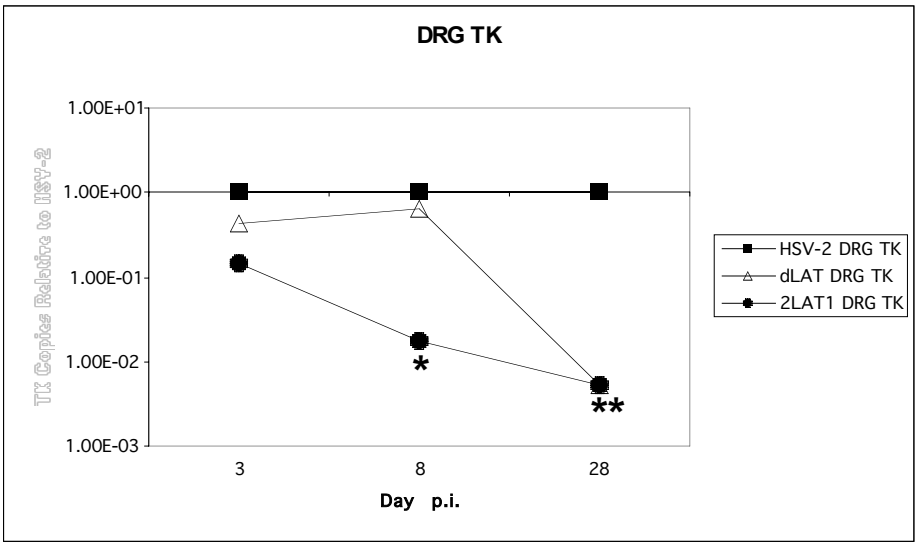
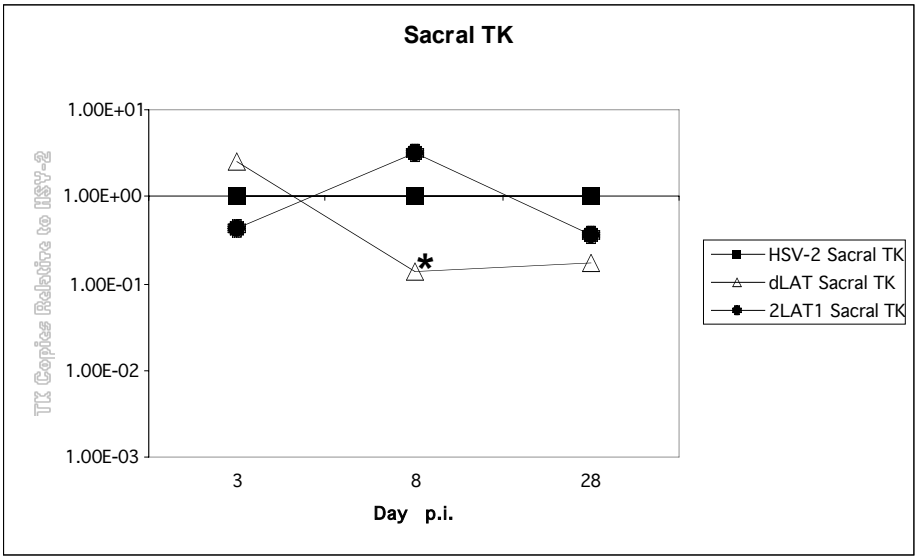
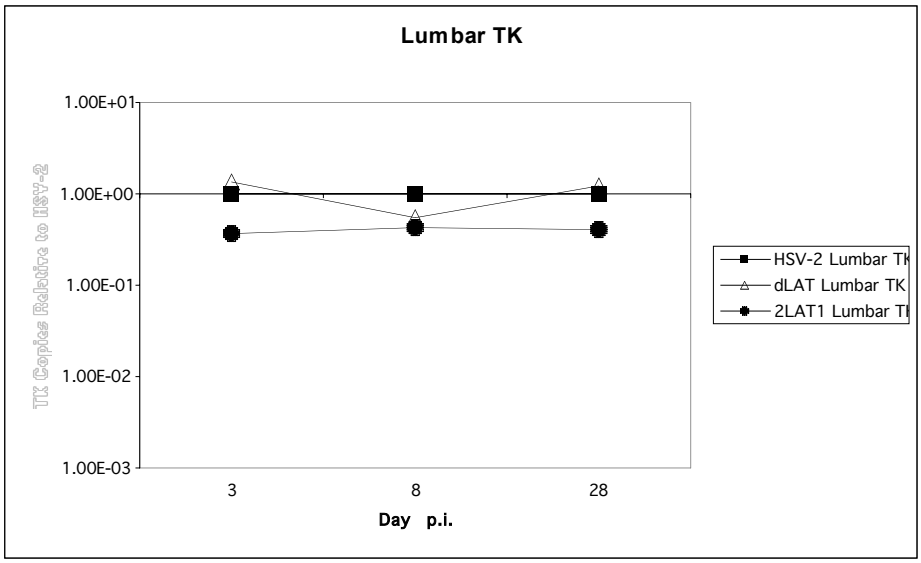

Lumbar ICP0
ICP0 Copies Relative to HSV-2
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
3
8
28
Day p.i.
*
HSV-2 Lumbar ICP0
dLAT Lumbar ICP0
2LAT1 Lumbar ICP0

Figure 35. TK Expression after Genital Inoculation.

RNA was extracted from DRG, sacral spinal cord, and lumbar spinal cord from intravaginally-infected guinea pigs sacrificed prior to the appearance of symptoms (Day 3), during acute infection (Day 8) and during latent infection (Day 28). RNA was normalized to the 18S ribosomal RNA. DNA quantities of ΔLAT and HSV-2/LAT1 were normalized to HSV-2 at each given time point and the RNA quantities were adjusted by the same factor. The quantities presented represent TK expression relative to the TK expression of HSV-2 at each given time point. * $p \leq 0.023$ compared to HSV-2 at the same time point. HSV-2 (n=6 at each time point), ΔLAT (n=3 at each time point), HSV-2/LAT1 (n=3 at each time point). *Only wild type HSV-2 was capable of replication in the DRG during latency; thus type-specific LAT regions are required for replication in the DRG during latency.*


DRG TK
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
TK Copies Relative to HSV-2
3
8
28
Day p.i.
*
**
HSV-2 DRG TK
dLAT DRG TK
2LAT1 DRG TK


Sacral TK
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
TK Copies Relative to HSV-2
3
8
28
Day p.i.
*
HSV-2 Sacral TK
dLAT Sacral TK
2LAT1 Sacral TK


Lumbar TK
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
TK Copies Relative to HSV-2
3
8
28
Day p.i.
HSV-2 Lumbar TK
dLAT Lumbar TK
2LAT1 Lumbar TK

Discussion

After intravaginal inoculation, HSV2/LAT1 resulted in a more severe acute infection than wild type HSV-2, although a more severe acute infection was not observed in the footpad infection. Footpad infections are typically less severe than genital infections in guinea pigs and differences in acute severity are not as apparent in the footpad model. After both routes of infection, HSV2/LAT1 was impaired for reactivation, consistent with previous studies characterizing HSV2/LAT1.

After inoculation of virus into the right hind footpad, HSV-2 and HSV-1 spread preferentially to different regions of the central nervous system. HSV-2 spread efficiently to the sacral spinal cord and less efficiently to the lumbar cord. HSV-1 spread efficiently to the lumbar spinal cord but was undetectable in the sacral spinal cord. Neither HSV-2 nor HSV-1 was capable of traversing the spinal cord to reach the contralateral DRG in detectable quantities.

By expressing HSV-1 LAT sequences in the context of HSV-2, HSV2/LAT1 gained the ability to spread more efficiently through the nervous system. The chimeric virus was present at much greater quantities in the ipsilateral DRG than either HSV-2 or HSV-1, suggesting that the HSV-1 LAT sequences improved the replicative efficiency of HSV-2 in the DRG. Although HSV2/LAT1 was present in the sacral spinal cord at levels similar to wild type HSV-2, the chimeric virus had greater quantities of viral DNA in the lumbar cord compared to HSV-2. The HSV-1 LAT sequences improved the ability of HSV-2 to either spread to or replicate in the lumbar spinal cord. The chimeric virus also spread efficiently to the contralateral DRG, although neither wild type virus had detectable levels of viral DNA in the contralateral DRG. This gain of function may have

resulted from increased replication in the spinal cord, allowing the virus to spread more easily to the contralateral DRG. However, HSV2/LAT1 viral DNA levels were similar to HSV-2 in the sacral spinal cord and similar to HSV-1 in the lumbar cord, which argues against increased replication in the spinal cord being the sole factor in the viral spread to the contralateral DRG. Therefore, the intact HSV-2 LAT region appears to limit the replication of HSV-2 within the DRG and also limit the spread of the virus.

After genital inoculation of HSV2/LAT1, the chimeric virus did not demonstrate the dramatic increase in viral spread through the central nervous system observed after footpad inoculation based on comparisons of viral DNA levels, although the acute lesion severity was increased compared to wild type HSV-2. This is likely due to differences in innervation between the footpad and the genitalia. Intravaginal inoculation provides immediate viral access to sensory neurons as well as autonomic neurons because of the dense population of nerve fibers terminating in the vaginal and cervical epithelium. In the footpad, sensory nerve endings terminate in the epithelium but autonomic nerve fiber endings are much more limited than in the cervicovaginal mucosa, although some sympathetic nerve endings are present in the footpad surrounding blood vessels and sweat glands. The virus therefore has much more limited access to autonomic neurons, particularly the parasympathetics, prior to reaching the central nervous system. The genital model provides information regarding the natural route of HSV transmission, including the sensory, autonomic, and central pathways. The footpad model limits initial viral entry to primarily sensory neurons of a single footpad, permitting observations to be made regarding spread originating from a single dominant pathway. The differences observed between the footpad and genital models suggest that the autonomic nervous

system plays an important role in viral entry, establishment of latency, and efficient reactivation of HSV-2 after genital transmission.

To more closely examine the effect of LAT on spread and replication of HSV-2, guinea pigs intravaginally-infected with HSV-2, ΔLAT, or HSV2/LAT1 were sacrificed at various time points during acute infection and latency to evaluate differences in viral DNA levels. Viral DNA comparisons between HSV-2 and ΔLAT (the LAT promoter deletion mutant) demonstrate that the HSV-2 LAT promoter region limits viral replication but does not limit the establishment of latency with HSV-2 infection. HSV2/LAT1 viral DNA levels after genital inoculation demonstrate that the limiting element may be present in both the HSV-1 and HSV-2 LAT regions, although this element for each virus likely functions in specific neuronal subtypes based on the observed differences in spread between the footpad model and the genital model and differences in innervation of the sites of inoculation. By limiting viral replication in specific neuronal subtypes while permitting lytic cycle gene expression in others, the wild type viruses are able to control viral spread and replication and effectively establish latency. By altering the HSV-2 LAT region, loss of viral control of viral gene expression in specific neuronal subtypes would permit dysregulated viral replication in neurons that would otherwise have limited lytic cycle gene expression.

Infected guinea pigs were also evaluated for RNA transcript expression of LAT, ICP0, and thymidine kinase (*tk*). The LAT promoter is required for LAT expression during latency but low levels of LAT are expressed during acute infection with ΔLAT, regardless of tissue evaluated. Reduced LAT expression correlated with a significant reduction in ICP0 and *tk* transcript expression compared to wild type HSV-2, suggesting

that the LAT region provides a regulatory function for ICP0 expression during latency that may have an impact on the ability of the virus to reactivate into a replicative cycle.

Type-specific LAT has different effects on transcript expression depending on tissue type. After footpad inoculation, HSV2/LAT1 expressed higher levels of LAT and ICP0 transcripts during latency compared to HSV-2, with a more pronounced effect in the sacral cord and the contralateral DRG than in the ipsilateral DRG or lumbar cord. After genital inoculation, the presence of HSV-1 LAT sequences in the HSV-2 genome reduced LAT transcript expression by 100-fold in the DRG and increased LAT expression in the spinal cord, although the trends throughout acute infection and latency remained similar. LAT expression increased exponentially through Day 8, after which LAT continued to increase at a slower rate into latency for both viruses. ICP0 and *tk* expression from HSV2/LAT1 were also significantly reduced in the DRG but the chimeric virus produced wild type levels of both transcripts in the spinal cord. Thus, type-specific LAT influences viral gene expression differently depending on tissue type and route of inoculation, potentially contributing to the ability of the wild type viruses to replicate in response to reactivating stimuli.

HSV is known to transport through and establish latency in sensory neurons. If the virus only used sensory pathways to reach the CNS, one would expect the relative loads in the sacral cord versus the DRG to be similar between the footpad and genital models since both of these sites of inoculation provide entry into the sensory nerve endings. After footpad infection, the amount of HSV-2 DNA found in the sacral cord was similar to the amount found in the DRG on the inoculated side. However after genital infection, there was significantly more viral DNA found in the sacral cord than in

the DRG, suggesting that after genital infection HSV-2 reaches the CNS via an additional route. The cervicovaginal epithelium permits viral entry into the parasympathetic nerve endings, which are not present in the footpad. Therefore, the additional route of entry into the CNS would be the parasympathetic pathway for HSV-2.

HSV-2 in the DRG after genital inoculation expressed significantly greater quantities of LAT and ICP0 per genome compared to that in the ipsilateral DRG in the footpad model. LAT and ICP0 were detected in the contralateral DRG although HSV-2 viral DNA was below the level of detection, suggesting inherent differences in the expression of viral transcripts depending on whether the virus reached the DRG from the periphery to reach the ipsilateral DRG, or from the spinal cord to reach the contralateral DRG. Virus reaching the DRG from the spinal cord expressed greater quantities of viral transcripts per viral genome. HSV-2/LAT1 expressed approximately the same quantities of LAT and ICP0 transcripts per viral genome in the DRG after genital inoculation and the contralateral DRG after footpad inoculation, and these quantities were 10-fold greater than in the ipsilateral DRG, suggesting that virus in the DRG after genital infection may have reached the DRG from the spinal cord since it expressed the greater quantities of viral transcripts per genome similar to the contralateral DRG after footpad infection. From the periphery, the virus is capable of infecting different types of neurons, some of which support productive infection and some of which support latent infection. From the spinal cord, the virus could be trans-synaptically targeted to those neurons that support latent infection and reactivation competence through specific types of neurons in the CNS, likely to be the parasympathetic neurons or interneurons that communicate between the sensory and parasympathetic neurons.

By comparing the footpad and genital models of HSV infection, it is evident that HSV-2 is transported through the nervous system via both sensory and parasympathetic pathways to reach the central nervous system. In both models, HSV-2 preferentially spreads to the sacral regions of the spinal cord while HSV-1 preferentially spreads to the lumbar regions of the spinal cord. The sacral and lumbar regions of the spinal cord contain the parasympathetic and sympathetic autonomic nuclei, respectively. The preferential spread into these regions of the spinal cord implies that the autonomic nervous system plays a distinctive role in viral spread and potentially in type-specific reactivation of HSV-1 and HSV-2. Viral gene expression differed depending on whether virus reached the DRG from the periphery or from the spinal cord, suggesting that viral spread through the central nervous system may be an integral part of the viral life cycle to efficiently establish reactivation-competent latency.

Chapter 6

Discussion

HSV-1 and HSV-2 are similar viruses in a number of ways. Primary infections with either virus can be subclinical or may produce characteristic vesicular lesions. During primary infections, the viruses are transported by retrograde axonal flow into the innervating sensory ganglia where they establish lifelong latency. In response to various stimuli, the viruses may periodically reactivate to cause recurrent lesions at or near the original site of inoculation. The viruses are also capable of causing central nervous system disease. Although it is generally accepted that the viruses rarely reach the nervous system to cause clinical symptoms, more recent reports suggest that the viruses may reach the central nervous system more commonly than thought.

There are several notable differences between HSV-1 and HSV-2 that are important for the pathogenesis of each individual virus. HSV-1 preferentially establishes latency in the trigeminal ganglia innervating the face and reactivates to cause recurrent cold sores and keratitis. HSV-2 preferentially establishes latency in the lumbosacral dorsal root ganglia and reactivates to cause recurrent genital herpes. The mechanisms for site-specific establishment of latency and reactivation are not understood. Upon reaching the central nervous system, HSV-1 and HSV-2 cause very different clinical syndromes. HSV-1 causes necrotizing hemorrhagic encephalitis with a high mortality rate, even with treatment, and results in neurological sequelae in most survivors. HSV-2 is associated with recurrent meningitis, which is rarely fatal and does not typically result in neurological sequelae. Other peripheral nervous system disorders have been described

for HSV-1 as well, including vestibular neuritis and Bell's Palsy. HSV-2 has been implicated in cases of sacral radiculopathy and myelitis in both immunocompetent and immunocompromised individuals. It is unclear why HSV-1 and HSV-2 have different capacities for causing central and peripheral nervous system disorders. At least some of the predilection for HSV-1 and HSV-2 to cause type-specific nervous system disease is simply related to their respective anatomical site-specific reactivation. Therefore, understanding the differences in type-specific establishment of latency and site-specific reactivation will also provide a greater understanding of nervous system complications related to each virus.

Acyclovir and its derivatives are currently used for treatment of HSV-1 and HSV-2 because of their highly specific mechanism of action and efficacy for inhibition of viral replication. When administered during acute primary infection, acyclovir may reduce the severity of clinical symptoms but it does not prevent the establishment of latent infection since latency is already largely established by the time symptoms appear. For recurrent peripheral disease, acyclovir must be administered daily to prevent genital herpes and the drug provides no protection once treatment is stopped. In central nervous system disease, many cases of encephalitis result in death or serious sequelae even with aggressive treatment. Currently, no vaccines are available for prevention of disease or establishment of latency.

A greater understanding of the mechanisms by which HSV-1 and HSV-2 establish latency and cause recurrent peripheral and CNS disease will enhance the ability of physicians to manage HSV-related syndromes and contribute to the development of antivirals and vaccine candidates. The clinical presentations of HSV CNS infections

suggest that neuronal spread of the virus may be differentially regulated between HSV-1 and HSV-2. The latency-associated transcript of HSV appears to provide regulatory control over the spread of the virus and establishment of latency in specific subtypes of neurons. Molecular analysis of the regulatory control of viral spread in the nervous system will also provide insight into methods for the prevention of CNS involvement following peripheral HSV infections. Therefore, I have characterized differences between HSV-1 and HSV-2 with respect to their abilities to spread into and through the central nervous system and establish latency in peripheral and central nervous system tissues. I have also evaluated the role of the latency-associated transcript in viral pathogenesis, and defined the specific region of LAT that confers type-specific reactivation.

Characterization of Differences Between HSV-1 and HSV-2 Spread, Replication and the Establishment of Latency in the Nervous System

HSV-1 and HSV-2 Spread to Different Regions of the Central Nervous System.

HSV-1 and HSV-2 spread preferentially to different regions of the central nervous system. HSV-1 DNA was found primarily in the lumbar spinal cord after both genital and footpad inoculations. After genital infection, significantly higher levels of HSV-1 DNA were found in the lumbar spinal cord compared to the sacral cord or the DRG. After footpad inoculation, HSV-1 DNA was found in the lumbar cord, but was below the limits of detection in the sacral cord. The quantities of HSV-2 DNA were significantly

greater in the sacral cord than in the DRG after genital inoculation, but similar between the two sites after footpad inoculation. The quantities of HSV-2 DNA were significantly greater in the sacral cord than in the lumbar cord after both genital and footpad inoculation. Regardless of the route of inoculation, HSV-2 finds its way to the sacral cord while HSV-1 preferentially transits to the lumbar cord.

The Autonomic Nervous System Plays a Role in Spread of HSV.

The cervicovaginal epithelium is densely innervated by sensory and autonomic nerve fibers. The cell bodies of sensory neurons reside in the lumbosacral DRG and have a single bifurcated axon with one branch extending to the periphery and the second branch extending to the spinal cord. The peripheral axonal branch extends its free nerve endings directly into the cervicovaginal epithelium. The autonomic nervous system consists of sympathetic and parasympathetic nerve fibers that also extend nerve endings directly into the cervicovaginal epithelium, with the sympathetic and parasympathetic pathways originating in the lumbar and sacral spinal cord, respectively. HSV-1 and HSV-2 are capable of entry into autonomic and sensory neurons and have direct access to these nerve endings during a genital infection. However, HSV-2 is unable to effectively establish latency in sympathetic neurons (75) while HSV-1 has been shown to transport through and establish latency in neurons of the sympathetic pathway (15, 34, 51, 101). HSV-1 is able to transport through parasympathetic fibers (51, 102) but latency has not been reported, although HSV-2 latency in parasympathetic neurons has been demonstrated (75).

After genital inoculation, HSV-2 DNA was found in greater quantities in the sacral cord compared to the lumbar cord, suggesting that HSV-2 is more efficient at spreading to or replicating in neurons residing in the sacral cord, which contains few neuronal cell bodies besides parasympathetic neurons and interneurons. Large quantities of HSV-1 DNA were found in the lumbar spinal cord but relatively little in the sacral cord, suggesting that HSV-1 is able to efficiently spread to or replicate in neuronal cell bodies residing in the lumbar cord, likely to be sympathetic neurons based on the neuronal pathways available to the virus. Since the viruses have access to autonomic nerve endings as well as sensory nerve endings in the cervicovaginal epithelium, HSV-1 and HSV-2 appear to use both autonomic and sensory pathways to enter into the nervous system. Based on findings presented here, it appears that HSV-1 may preferentially use the sympathetic pathway while HSV-2 may preferentially use the parasympathetics, in addition to the sensory neuronal pathway.

The innervation of the footpad is quite different from the cervicovaginal epithelium. Although some autonomic nerve fibers are present, mostly sympathetic fibers surrounding small blood vessels and sweat glands, the footpad is innervated primarily by sensory nerve endings. The footpad model was used to evaluate viral spread in addition to the genital model to attempt to distinguish between sensory and autonomic pathways of transport into the spinal cord. Inoculation into the footpad directs viral entry primarily to sensory nerve endings, limiting entry into the autonomic pathways, particularly the parasympathetics. Footpad inoculation provided a 1-2 log decrease in viral DNA in the nervous system tissues compared to the genital model, with a more dramatic difference apparent in the spinal cord regions than in the DRG for HSV-2.

After genital inoculation, the sacral cord contained greater quantities of HSV-2 DNA than the DRG, but after footpad inoculation the quantities of DNA found in the DRG and sacral cord were similar. These findings suggest that the parasympathetic pathway may be more important than the sensory pathway for HSV-2 to reach the central nervous system. If viral spread were only through the sensory neurons into the spinal cord, one would expect the footpad and genital models to be similar with respect to relative amounts of HSV-2 DNA in the sacral cord and DRG. With HSV-1, there was an approximately 10-fold greater quantity of viral DNA in the lumbar cord than the DRG after both routes of inoculation. Since sympathetic nerve fibers innervate the blood vessels of the footpad and the virus is inoculated into the foot by needle injection, the virus would have access to sympathetic nerve endings by both genital and footpad routes of inoculation allowing similar relative differences between the lumbar cord and the DRG in both models. However, HSV-1 viral DNA was at a very low level in the sacral cord after genital inoculation and undetectable after footpad inoculation, demonstrating that HSV-1 is restricted in the sacral cord, possibly due to an inability to spread to or replicate in parasympathetic neurons.

The work presented here demonstrates that HSV-1 and HSV-2 have different capabilities of entering or replicating in neurons of the lumbar and sacral spinal cord. Given the opportunity to enter equivalently into sensory and autonomic nerve endings via the genital route of inoculation, HSV-2 spreads more efficiently to the sacral spinal cord while HSV-1 spreads more efficiently to the lumbar spinal cord. Limiting entry into the parasympathetic system by inoculating the virus into the footpad results in equivalent quantities of HSV-2 DNA in the sacral cord and the DRG, which suggests that the

parasympathetic pathway is of critical importance for HSV-2 viral spread to the spinal cord. HSV-1 had comparable relative quantities of viral DNA in the lumbar cord and DRG after both routes of inoculation. HSV-1 could potentially have reached the lumbar cord via the sympathetic pathway or the sensory pathway and it is not possible to distinguish the specific route of transport using these models. However, HSV-1 is clearly restricted in the sacral spinal cord regardless of route of infection and HSV-2 appears to be restricted in the lumbar cord, although not to as great an extent as HSV-1 in the sacral cord.

Viral Spread from the Periphery Differs from Viral Spread from the Spinal Cord.

The latency-associated transcript (LAT) is the hallmark of HSV latency. It is widely accepted that the detection of the LAT intron denotes latency in infected tissues. After footpad inoculation of HSV-1 and HSV-2, LAT expression was detected in the contralateral DRG although viral DNA was below the limits of detection by Taqman PCR assay. Thus, small quantities of HSV-1 and HSV-2 DNA reached the contralateral DRG but were unable to replicate efficiently to produce quantifiable amounts of viral DNA. To reach the contralateral DRG after unilateral footpad inoculation, the virus must have transported retrogradely through the sensory neurons into the spinal cord and into the contralateral DRG. These small quantities of viral DNA that were below the limits of detection expressed LAT at levels approaching the levels expressed in the spinal cord and ipsilateral DRG, both of which contained significantly greater quantities of viral DNA. Additionally, the undetectable quantity of HSV-2 DNA in the contralateral DRG also expressed ICP0 at levels similar to that expressed in the ipsilateral DRG and sacral spinal

cord (each of which contained over 1000 copies of HSV-2 DNA per 250 ng of total DNA in the tissues) although ICP0 expression was not detected in the HSV-1-infected contralateral DRG. These findings suggest that virus that reaches the DRG neurons from the spinal cord behaves differently than virus that reaches the DRG from the periphery. Centrally-transported HSV-2 failed to replicate in the DRG neurons but expressed LAT and ICP0, suggesting latency and reactivation competence. Centrally-transported HSV-1 failed to replicate in the DRG neurons and expressed LAT but did not express ICP0, which implies that the virus was able to establish latency but may not have been competent for reactivation. The most likely explanation for these findings is that virus transported from the spinal cord selectively reaches specific types of neurons that support latency rather than productive infection. From the periphery, the virus is capable of infecting different types of neurons, some of which support productive infection and some of which support latent infection. From the spinal cord, the virus is trans-synaptically targeted preferentially to those neurons that support latent infection, resulting in LAT expression without viral replication and detectable levels of DNA.

The Role of LAT in Viral Spread and Pathogenesis.

HSV-2/LAT1, the chimeric HSV-2 virus expressing the LAT from HSV-1, was compared to HSV-2 and HSV-1 in the footpad model of HSV infection to evaluate the role of type-specific LAT in viral spread. ΔLAT, the LAT promoter-deleted mutant of HSV-2 that does not express the LAT transcript, was compared to HSV-2 in the genital model of infection to determine the effect of the HSV-2 LAT promoter region on

pathogenesis, replication, and viral gene expression. HSV-2/LAT1 was also compared to HSV-2 in the genital model to evaluate differences between the LAT regions of HSV-1 and HSV-2 with regard to their effects on viral replication and gene expression.

The LAT Limits the Severity of Acute Disease in the Genital Model.

In all experiments with HSV-2/LAT1 and ΔLAT, a moderate increase in the severity of acute disease was observed. Although the differences between the LAT mutant viruses and wild type viruses were not statistically significant in any individual experiment, the observed increases in acute lesion severity were consistently apparent across all experiments. In addition, a greater percentage of animals infected with HSV-2/LAT1 and ΔLAT had observable increases in urinary tract dysfunction compared with those infected with wild type HSV-2. An unrealistic quantity of guinea pigs would be required to obtain statistical significance due to the variability of the animals. Without statistical significance, it is difficult to make a firm conclusion regarding changes in disease severity; however the repeatability of these observations throughout numerous animal experiments demonstrates that increased severity with LAT mutants is a real finding. Therefore, the LAT region appears to be involved in limiting the severity of disease.

The LAT Region Confers Type-Specific Reactivation.

After both genital and footpad inoculations, HSV-2 reactivates efficiently to cause recurrent lesions while HSV-1 reactivates with a significantly lower frequency. Without LAT expression due to the deletion of the LAT promoter, ΔLAT fails to reactivate

efficiently and demonstrates a recurrence frequency similar to that of HSV-1. After both genital and footpad inoculations, the chimeric virus HSV-2/LAT1 reactivates at a significantly lower frequency than HSV-2 but at a slightly, but not significantly, higher frequency than HSV-1. Therefore, LAT expression is required for efficient reactivation of HSV-2. Furthermore, the region of HSV-2 LAT including the promoter, exon 1, and the LAT intron are required for the characteristic type-specific high recurrence frequency of HSV-2. These results presented here are consistent with previously published data (38, 49, 114).

The LAT Region Influences Neurotropism and Latent Viral Load.

To evaluate the role of LAT in viral replication and the establishment of latency, the LAT promoter deletion mutant of HSV-2 was compared to wild type HSV-2 in the guinea pig genital model during early transcription prior to the appearance of symptoms (Day 3), during the peak of acute infection (Day 8), and during latency (Day 28). In the DRG, ΔLAT produced significantly greater quantities of viral DNA during the acute infection but established a latent viral load similar to HSV-2. However in the sacral spinal cord, the quantities of ΔLAT viral DNA were significantly higher than HSV-2 at all time points during acute and latent infection. These results suggest that one function of LAT is to limit viral DNA replication, which supports the hypothesis that the LAT region limits severity of disease. In the lumbar spinal cord, no significant effect was observed from the deletion of the LAT promoter. Deletion of the LAT promoter produced different effects on viral DNA load in the DRG, the sacral spinal cord, and the

lumbar cord, demonstrating that the HSV-2 LAT function is dependent on the cellular environment of neurons within each of these regions of the nervous system.

To evaluate differences between HSV-1 and HSV-2 LAT regions with respect to their effects on viral replication and the establishment of latency, HSV-2/LAT1 was compared to HSV-2 in the genital model of infection. HSV-2/LAT1 produced significantly less viral DNA than HSV-2 in the DRG. In the spinal cord, HSV-2/LAT1 produced a small decrease in the sacral cord and a small increase in the lumbar cord compared to HSV-2. The HSV-1 LAT region appears to limit viral replication in the DRG to a greater extent than the HSV-2 LAT region. Although the difference between the LAT regions is not as pronounced in the spinal cord as in the DRG, HSV-1 LAT appears to limit replication of the virus better in the sacral than in the lumbar region. As with HSV-2, the HSV-1 LAT region also appears to function differently in various regions of the nervous system, supporting a neuron-specific function for LAT.

To evaluate differences between the LAT regions of HSV-1 and HSV-2 with respect to their effects on viral spread through the nervous system, HSV-2/LAT1 was compared to HSV-2 and HSV-1 in the footpad model of infection. After footpad inoculation, HSV-2/LAT1 viral DNA quantities were greater than HSV-2 in the ipsilateral DRG. Expressing the HSV-1 LAT in the context of HSV-2 either enhanced the replicative capabilities of the virus or the ability of the virus to spread to the ipsilateral DRG after footpad infection, although increased DRG replication was not observed after genital infection. HSV-2/LAT1 also produced quantifiable amounts of DNA in the contralateral DRG, although neither parent virus was capable of replicating to a detectable level on the contralateral side, suggesting an enhancement of either spread

to or replication in the contralateral DRG. In the spinal cord, the viral DNA levels of HSV-2/LAT1 were similar to HSV-2 in the sacral cord and similar to HSV-1 in the lumbar cord (10-fold higher than HSV-2), implying that the HSV-1 LAT region permitted HSV-2/LAT1 to replicate better in the lumbar cord without significant effects in the sacral cord. These results support the hypothesis that the LAT region influences viral spread and the establishment of latency through an effect on viral replication, which differs depending on the region of the nervous system.

Without the LAT promoter, HSV-2 replicates to a higher titer in the DRG and the sacral cord after genital infection but no effect is observed in the lumbar cord, suggesting that a functional element in the LAT promoter inhibits replication in the DRG and sacral cord. HSV-1 LAT placed into HSV-2 inhibits replication in the DRG and sacral cord as well, but enhances replication in the lumbar cord. These results provide evidence that the LAT regions of HSV-2 and HSV-1 function differently in the DRG and the sacral and lumbar regions of the spinal cord, implying differences in the cellular environments surrounding the virus.

The LAT Region Influences Viral Gene Expression Differently in the DRG and Spinal Cord.

ΔLAT and HSV-2/LAT1 were compared to HSV-2 to determine the effects of the LAT region on viral gene expression after genital inoculation. Viral gene expression of HSV-2/LAT1 was also compared to HSV-2 after footpad inoculation. As expected, ΔLAT expressed very low levels of LAT in all tissues evaluated. ΔLAT also expressed significantly lower levels of ICP0 compared to HSV-2, suggesting that LAT expression,

or a *cis*-acting element within the LAT promoter, may be a requirement for ICP0 expression. After footpad inoculation, LAT and ICP0 transcript expression per genome by HSV-2/LAT1 was more similar to HSV-1 than HSV-2, as shown in Table 3 in Chapter 5. Thus, the LAT region regulates not only expression of LAT, but also of ICP0.

After genital inoculation, HSV-2/LAT1 expressed lower levels of LAT and ICP0 in the DRG compared to HSV-2. In the sacral cord, HSV-2/LAT1 expressed higher levels of LAT and ICP0 compared to HSV-2 during the acute infection but wild type levels during latency. In the lumbar cord, HSV-2/LAT1 expressed higher levels of LAT but expressed ICP0 at levels similar to wild type HSV-2 during both acute and latent infection. These results indicate that HSV-1 and HSV-2 LAT regions function differently in the various regions of the nervous system, which implies differences in the types of neurons in which the viruses reside in each region of the nervous system.

Thymidine kinase (*tk)* transcript expression also varied by tissue region. HSV-2/LAT1 did not have detectable levels of *tk* expression in the DRG, although HSV-2 continued to express *tk* into latency, but the chimeric virus expressed wild type HSV-2 levels of *tk* in the spinal cord after genital infection. Not only does this demonstrate differences in the cellular environments of infected cells in the different regions of the nervous system, but also suggests that the limited recurrences observed during HSV-2/LAT1 latency may have originated in the spinal cord rather than the DRG.

The cellular environments surrounding the viruses in various regions of the nervous system appear to have substantially different effects on the performance of the LAT regions of HSV-1 and HSV-2, permitting differential viral gene expression and replication. These differential effects, in turn, influence the establishment of latency in

specific types of neurons in the DRG and the spinal cord. The overall effect of the LAT region is to direct HSV-1 and HSV-2 to appropriate neuronal pathways that permit each virus to establish latent infection in specific types of neurons that support reactivation in response to stimuli that enhance transmission of each virus to a new host. For HSV-2, transmission may be facilitated by reactivation from a site of latency in response to neuronal stimulation associated with sexual activity, which includes both sensory and autonomic pathways. Although the autonomic nervous system has been largely ignored in studies on HSV latency, the work presented here supports a significant role for the autonomic nervous system in viral spread, replication, establishment of latency and reactivation.

Characterization of the LAT Region that Confers the Type-Specific Reactivation Phenotype to HSV-2.

HSV-2/LAT1 failed to reactivate efficiently in the guinea pig genital model of HSV, in the current studies and in a previous report (114), while its rescuant reactivated with a wild type HSV-2 phenotype. Therefore, the LAT region confers the type-specific reactivation phenotype to HSV-2.

To determine whether the LAT promoter or the LAT sequence downstream of the promoter were responsible for conferring type-specific reactivation, additional chimeric viruses HSV2-LAT-P1 and HSV2-LAT-S1 were constructed and characterized *in vitro* and in the guinea pig genital model of infection. Based on results from these two chimeric viruses and published literature on the HSV-1 LAT region, a third chimeric

virus was constructed, replacing the HSV-2 LAT exon 1 from the TATA box to the 5' splice site of the LAT intron with the corresponding region from HSV-1.

LAT Sequence Confers the Type-Specific Reactivation Phenotype and Contributes to Disease Severity.

HSV2-LAT-P1 is a chimeric HSV-2 virus that expresses the native HSV-2 LAT sequences under the control of the HSV-1 LAT promoter (LAT promoter swap). HSV2-LAT-S1 expresses HSV-1 LAT sequences downstream of the native HSV-2 LAT promoter in an HSV-2 backbone (LAT sequence swap). HSV2-LAT-P1 reactivated with a wild type HSV-2 recurrence phenotype, although the acute infection was attenuated in both lesion severity and urinary tract dysfunction compared to wild type HSV-2. HSV2-LAT-S1 failed to reactivate efficiently after producing a more severe acute infection characterized by increased lesion severity and a greater percentage of guinea pigs experiencing urinary tract dysfunction. Although HSV2-LAT-S1 did not reactivate efficiently, most recurrences that did occur caused severe progressive disease and were frequently fatal. The rescuant of HSV2-LAT-S1 demonstrated a wild type HSV-2 phenotype with regard to both acute disease severity and reactivation frequency. Thus the LAT sequence, rather than the LAT promoter, provides the essential elements for efficient genital reactivation of HSV-2 and limits the severity of disease.

Increased mortality was not observed after infections with HSV-2/LAT1, which contains the HSV-1 LAT region including the promoter, exon 1, and most of the intron. HSV2-LAT-S1 contains the same portion of the HSV-1 LAT except for the promoter. Increased or decreased virulence in previous reports on HSV-1 LAT region deletion

mutants typically depended on the size of the deletion and the species infected (79, 84), suggesting that the LAT region has multiple functional elements and that specific neuronal factors may interact with LAT sequences to regulate viral replication and virulence. An interaction between the HSV-2 LAT promoter and the HSV-1 LAT sequence, mediated by specific neuronal factors, may have contributed to the increased mortality and fatal recurrences observed with HSV2-LAT-S1 infection. Regulatory elements at the mutation junction appear to be critical for limiting virulence.

LAT Exon 1 Confers the Type-Specific Reactivation Phenotype to HSV-2.

HSV2-LAT-E1 is a chimeric HSV-2 virus that has the HSV-2 LAT exon 1 from the TATA box to the 5' splice site of the LAT intron replaced by the corresponding region of HSV-1 (LAT exon swap). HSV2-LAT-E1 produced an acute infection similar to its rescuant and wild type HSV-2 but failed to reactivate efficiently, while a wild type reactivation frequency was restored in the rescuant. Therefore, the region that lies between the TATA box and the 5' splice site of the LAT intron, or LAT exon 1, confers the type-specific reactivation phenotype to HSV-2.

The failure to reactivate did not result from a failure to replicate or establish latency, as viral DNA levels were similar between HSV2-LAT-E1 and HSV-2 during acute infection and latency. The reactivation phenotype was also not related to differences in viral gene expression of LAT, ICP0, or thymidine kinase, since transcription levels were similar between the viruses during acute and latent infection. However, HSV2-LAT-E1 preferentially established latency in A5+ neurons, a subset of nociceptive neurons in which HSV-1 also preferentially establishes latency. These

results suggest that reactivation competence relies on the type of neuron in which the latent virus resides.

Differences Between HSV2-LAT-S1 and HSV2-LAT-E1.

There were two primary differences between HSV2-LAT-S1 and HSV2-LAT-E1 that warrant discussion. Both of these chimeric viruses reactivated with decreased efficiency compared to wild type HSV-2, but they differed with respect to their mortality rate and the neuronal subtype in which they established latency. Although a greater percentage of guinea pigs infected with HSV2-LAT-E1 experienced urinary tract dysfunction during acute disease, increased mortality was not observed with this chimeric virus as in HSV2-LAT-S1 infection. Fatal recurrences were also not observed in HSV2-LAT-E1 infection.

In collaboration with Todd Margolis of The Proctor Foundation (University of California, San Francisco), HSV2-LAT-S1 and HSV2-LAT-E1 were evaluated for establishment of latency in A5+ and KH10+ neurons. HSV-1 preferentially establishes latency in A5+ neurons, while HSV-2 establishes latency in KH10+ neurons. HSV-2/LAT1 also establishes latency preferentially in A5+ neurons, implicating neuronal subtypes in reactivation competence. Both HSV2-LAT-S1 and HSV2-LAT-E1 were expected to establish latency in A5+ neurons based on their reactivation frequencies. HSV2-LAT-E1 established latency in A5+ neurons as expected (its rescuant preferred KH10+ neurons). However, HSV2-LAT-S1 established latency preferentially in KH10+ neurons, like wild type HSV-2, suggesting that the failure of HSV2-LAT-S1 and HSV2-LAT-E1 to reactivate occurred by different mechanisms.

HSV2-LAT-S1 contains the region of HSV-1 from a PvuI restriction enzyme site just downstream of the TATA box to an XhoI site in the LAT intron (refer to Figure 45 on page 27). HSV2-LAT-E1 contains the region of HSV-1 from the TATA box to the 5' splice site of the LAT intron. Therefore, the differences between the two chimeric viruses are a portion of the LAT intron and a small region between the TATA box and the PvuI site.

Several experiments have excluded the LAT intron itself as the critical region for reactivation or virulence, including deletion mutations (30, 44), transgenic mouse studies (60, 106), and intron destabilization (3, 71, 74). HSV2-LAT-E1 also excludes the LAT intron as the critical region since this chimeric virus contains the intact HSV-2 LAT intron, but does not reactivate efficiently. Therefore, the region between the TATA box and the PvuI restriction site is implicated in the differences in neuronal subtype and mortality observed between HSV2-LAT-E1 and HSV2-LAT-S1.

HSV-2 has 49 base pairs between the TATA box and the PvuI site, while HSV-1 has 28 base pairs (Figure 36). The 28 base pairs are nearly identical and HSV-2 has an additional 21 base pairs just upstream of the PvuI site, suggesting that these additional 21 bases may be of critical importance.

RNA folding was evaluated as a potential mechanism for the differences between HSV2-LAT-S1 and HSV2-LAT-E1. Using Mfold (67, 115), HSV-2 and HSV-1 LAT exon 1 sequences were compared, along with HSV2-LAT-S1 exon 1. HSV2-LAT-E1 contains the entire HSV-1 LAT exon 1, so if RNA folding of this region influenced virulence or neuron-specific establishment of latency, one would expect HSV2-LAT-E1 to behave like HSV-1. Indeed, HSV2-LAT-E1 establishes latency preferentially in A5+

neurons, as does HSV-1, and HSV2-LAT-E1 produces an acute infection similar to HSV-1 (which is also similar to HSV-2). However, there are minimal differences in the folding patterns of the RNA between HSV2-LAT-E1 and HSV2-LAT-S1. Because the only difference in the sequences of this region is less than 50 bases at the leading end of the RNA, the only prominent structural change is a small bulge representing the 21-base addition in HSV2-LAT-S1 compared to HSV2-LAT-E1. Therefore, it is unlikely that the structure of the folded RNA in this region is responsible for the differences observed between HSV2-LAT-S1 and HSV2-LAT-E1.

The entire region between the TATA box and the LAT intron splice site was analyzed for potential transcription factor binding sites in both HSV-1 and HSV-2 using several programs available on the TransFac transcription factor website, with the most extensive results obtained with AliBaba2 (35). In the small region between the TATA box and the PvuI site, both HSV-1 and HSV-2 have consensus sequences for transcription factors WT1 and Sp1 (Figure 36). HSV-2 also contains a consensus sequence for transcription factor AP-2α and an additional Sp1 binding site within the 21 base pairs missing in HSV-1.

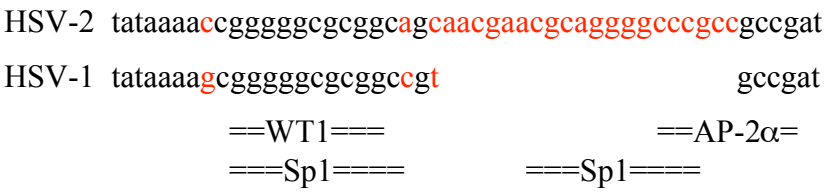


Figure 36. Transcription Factor Binding Sequence Sites in HSV-2 and HSV-1.

HSV-2 and HSV-1 transcription factor binding sites between the TATA box and the PvuI restriction enzyme site, identified by AliBaba2.1 (35) on the TransFac website (http://www.gene-regulation.com/pub/databases.html). Sequence that differs is shown in red.

This LAT region is highly enriched for GC and Sp1 binding sites are found repeatedly throughout the LAT exon 1, so an additional Sp1 site is not likely to dramatically impact the enhancer activity of the region. A putative AP-2α site also was located just downstream of the PvuI site in HSV-1, although the sequence does not conform to published consensus sequences for the transcription factor. AP-2α is phosphorylated by protein kinase A (PKA) or calcium-dependent kinases, which have been implicated in activating the productive cycle of HSV (20, 93, 108). The LAT exon 1 has known enhancer activity (5, 57) and may act as an enhancer for ICP0 (14), which permits the activation of viral genes associated with the productive cycle. It is possible that the AP-2α transcription factor may play a role in the increased mortality of HSV2-LAT-S1 by binding the LAT exon 1 and up-regulating productive cycle gene expression and replication, which would also result in the increased viral DNA load identified after HSV2-LAT-S1 infection. This AP-2α site could also enhance viral replication selectively in specific neurons, leading to cell death in A5+ neurons for HSV2-LAT-S1, which has the consensus sequence, and permitting latency in A5+ neurons for HSV2-LAT-E1, which does not have the site. It is possible that the AP-2α binding site could account for the differences between HSV2-LAT-S1 and HSV2-LAT-E1, although other as yet unidentified factors may also be involved. Additional studies would be required to ascertain the validity of AP-2α binding to the sequence and regulating the productive cycle of HSV.

Summary of Results

HSV-2 and HSV-1 have been characterized with respect to differences in viral spread, replication, viral gene expression, and reactivation. Several chimeric viruses and a deletion virus have also been used in these studies to determine the role of LAT in spread, replication, viral gene expression, and reactivation. Each of these viruses has provided a piece of the puzzle of HSV latency and reactivation, and has suggested additional studies to bring us closer to the solution to the puzzle. Taken together, the wild type and chimeric viruses suggest that certain aspects of the current model of HSV spread and latency should be reconsidered, taking into consideration the involvement of the spinal cord and the autonomic nervous system.

Figure 37 provides a graphic summary of the LAT region effects on establishment of latency and reactivation observed in our studies with HSV-1, HSV-2, the chimeric viruses, and the LAT promoter mutant. HSV-2, HSV-1, the chimeric viruses, and the promoter-deletion virus all produced fairly similar acute infections, although small but reproducible increases in severity were observed with the LAT mutants, with the exception of highly virulent HSV2-LAT-S1 that produced significantly more severe disease during both acute infection and recurrences. Thus, elements within the LAT region are important to limit severity of disease. HSV-2 reactivated efficiently in the guinea pig genital model while HSV-1 demonstrated a significantly reduced reactivation frequency. The LAT promoter-deletion virus demonstrated that elements within the promoter region are critical for reactivation, although the chimeric virus HSV2-LAT-P1 (the promoter swap) showed that the promoter region has little to do with type-specific reactivation.

The LAT promoter-deleted HSV-2 virus demonstrated that elements within the LAT promoter limit viral replication in the DRG and sacral spinal cord, since ΔLAT produced greater quantities of viral DNA in the DRG and sacral cord compared to wild type HSV-2. HSV-2/LAT1 and the promoter chimera, HSV2-LAT-P1, demonstrated that the HSV-1 LAT promoter up to the PvuI restriction site was able to limit replication in the sacral cord to a greater extent than the HSV-2 promoter, as shown by the reduced viral DNA in the sacral cord. Therefore, the promoter regions of HSV-1 and HSV-2 contribute to differential replication efficiency in the sacral cord. Both HSV-1 and HSV-2 have cAMP response elements (CRE) just upstream of the TATA box. The HSV-1 CRE is important for inducible reactivation but HSV-2 CRE has yet not been analyzed. It is possible that the CRE in HSV-1 and HSV-2 are differentially regulated in different types of neurons, permitting LAT expression or productive cycle gene expression dependent upon modifying cofactors within specific cell types. It is also likely that other neuronally-responsive elements are present in the promoter region, contributing to type-specific differences in viral replication in the sacral cord.

The region immediately surrounding the PvuI site of both HSV-1 and HSV-2 appears to have a dramatic impact on the virus, based on differences observed with HSV2-LAT-S1 and HSV2-LAT-E1, as discussed previously, as well as with HSV2-LAT-P1. HSV2-LAT-S1 caused more severe disease then HSV2-LAT-E1 (which was similar to wild type in severity) while the disease caused by HSV2-LAT-P1 was less severe. Just upstream of the PvuI site, HSV-2 contains an AP-2α transcription factor consensus sequence that is missing in HSV-1. However, a transcription factor binding site analysis identified a putative AP-2α binding site just downstream of the PvuI site in HSV-1,

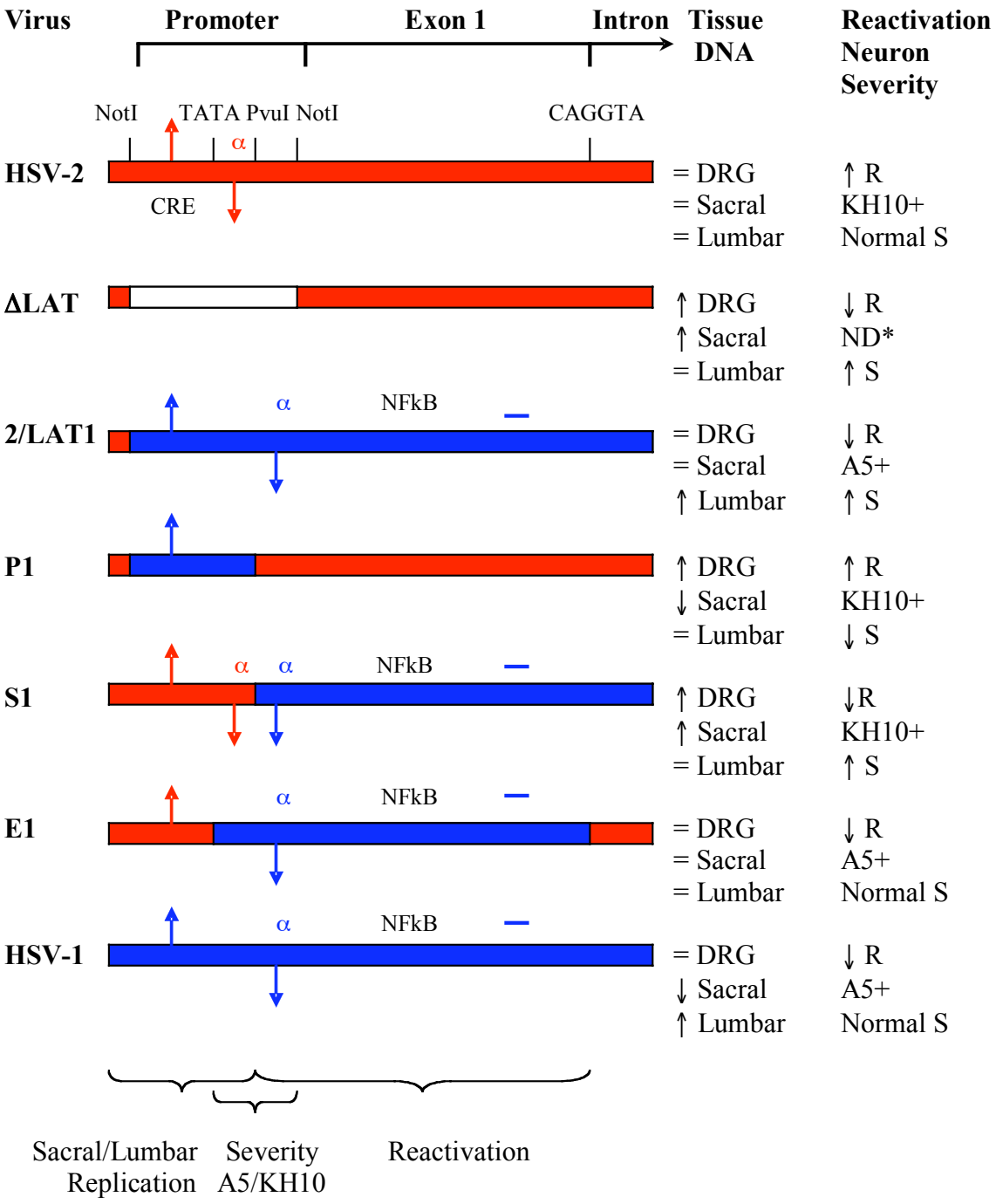
although it does not conform to published consensus sequences for AP-2α so it may have different properties than the site found in HSV-2. HSV2-LAT-E1 contains the nonconsensus AP-2α binding sequence from HSV-1, HSV2-LAT-S1 contains the AP-2α sites from both HSV-2 and HSV-1, and HSV2-LAT-P1 contains neither site, which correlates with the degree of disease severity if the AP-2α binding site contributes to severity of disease. The AP-2α transcription factor could also potentially interact with neuron-specific cofactors to modify the response of the CRE or other elements within the promoter region. Whether the putative binding sites for AP-2α impact the severity of disease remains to be evaluated. Regardless, this region near the PvuI restriction site appears to contain critical elements contributing to severity of disease and possibly to the differences in neuronal subtype for establishment of latency.

The region of LAT that lies between the PvuI restriction site and the 5' splice site of the LAT intron regulates reactivation competence. The HSV-1 LAT exon 1 contains three putative NFkB binding sites while none were identified in HSV-2 exon 1. In addition, a miRNA is reportedly generated by HSV-1 from within LAT exon 1, although this miRNA is not evident in HSV-2. Interestingly, the chimeric viruses that establish latency in A5+ neurons and reactivate inefficiently all contain the sequences containing both the NFkB binding sites and the miRNA sequence. HSV2-LAT-S1 also contained these sequences and reactivated inefficiently although it did not preferentially establish latency in A5+ neurons. This discrepancy could potentially be explained by the presence of the putative AP-2α sites from both HSV-1 and HSV-2, which may have altered the replicative and spread characteristics of the virus to an extent that prevented effective

establishment of latency in A5+ neurons, regardless of the presence of the NFkB sites and the miRNA.

Figure 37. Virus LAT region effects on establishment of latency and reactivation.

LAT region including the promoter (NotI-NotI) and Exon 1 (TATA-CAGGTA), showing regions of replacement in the chimeric viruses relative to HSV-2 and HSV-1. HSV-2 sequences and elements are shown in red and HSV-1 sequences and elements are shown in blue. **Tissue DNA** represents the amount of viral DNA found in the DRG, sacral cord, and lumbar cord relative to HSV-2. ↑ **R** represents normal HSV-2 reactivation frequency, which is higher than HSV-1, and ↓ **R** denotes a lower frequency of reactivation, similar to HSV-1. **Neuron** identifies the sensory neuronal subtype in which latency is established (A5+ or KH10+). **Normal S** indicates the typically observed level of disease severity for HSV-2 and HSV-1, ↑ **S** indicates increased disease severity compared to HSV-2 and HSV-1, and ↓ **S** indicates decreased disease severity compared to HSV-2 and HSV-1. Colored arrows pointing up in the promoter region represent the cAMP response element, red for HSV-2 CRE and blue for HSV-1 CRE. Colored arrows pointing down represent putative AP-2α transcription factor binding sites, red for HSV-2 and blue for HSV-1. NFkB consensus sequences are designated and blue dash to the right of NFkB represents miRNA produced from HSV-1 LAT region. NotI to PvuI region contributes to the ability to reactivate (although not type-specific reactivation) and the efficiency of replication in the sacral and lumbar cord. TATA to NotI (or the region immediately surrounding the PvuI site) contributes to severity of disease and possibly to A5+ and KH10+ neuron differences. PvuI to CAGGTA regulates type-specific reactivation.


Virus
Promoter
Exon 1
Intron
Tissue DNA
Reactivation Neuron Severity
NotI
TATA PvuI NotI
CAGGTA
α
HSV-2
CRE
= DRG
= Sacral
= Lumbar
↑ R
KH10+
Normal S
ΔLAT
↑ DRG
↑ Sacral
= Lumbar
↓ R
ND*
↑ S
2/LAT1
NFkB
= DRG
= Sacral
↑ Lumbar
↓ R
A5+
↑ S
P1
↑ DRG
↓ Sacral
= Lumbar
↑ R
KH10+
↓ S
S1
↑ DRG
↑ Sacral
= Lumbar
↓R
KH10+
↑ S
E1
= DRG
= Sacral
= Lumbar
↓ R
A5+
Normal S
HSV-1
↓ DRG
↓ Sacral
↑ Lumbar
↓ R
A5+
Normal S
Sacral/Lumbar Replication
Severity A5/KH10
Reactivation

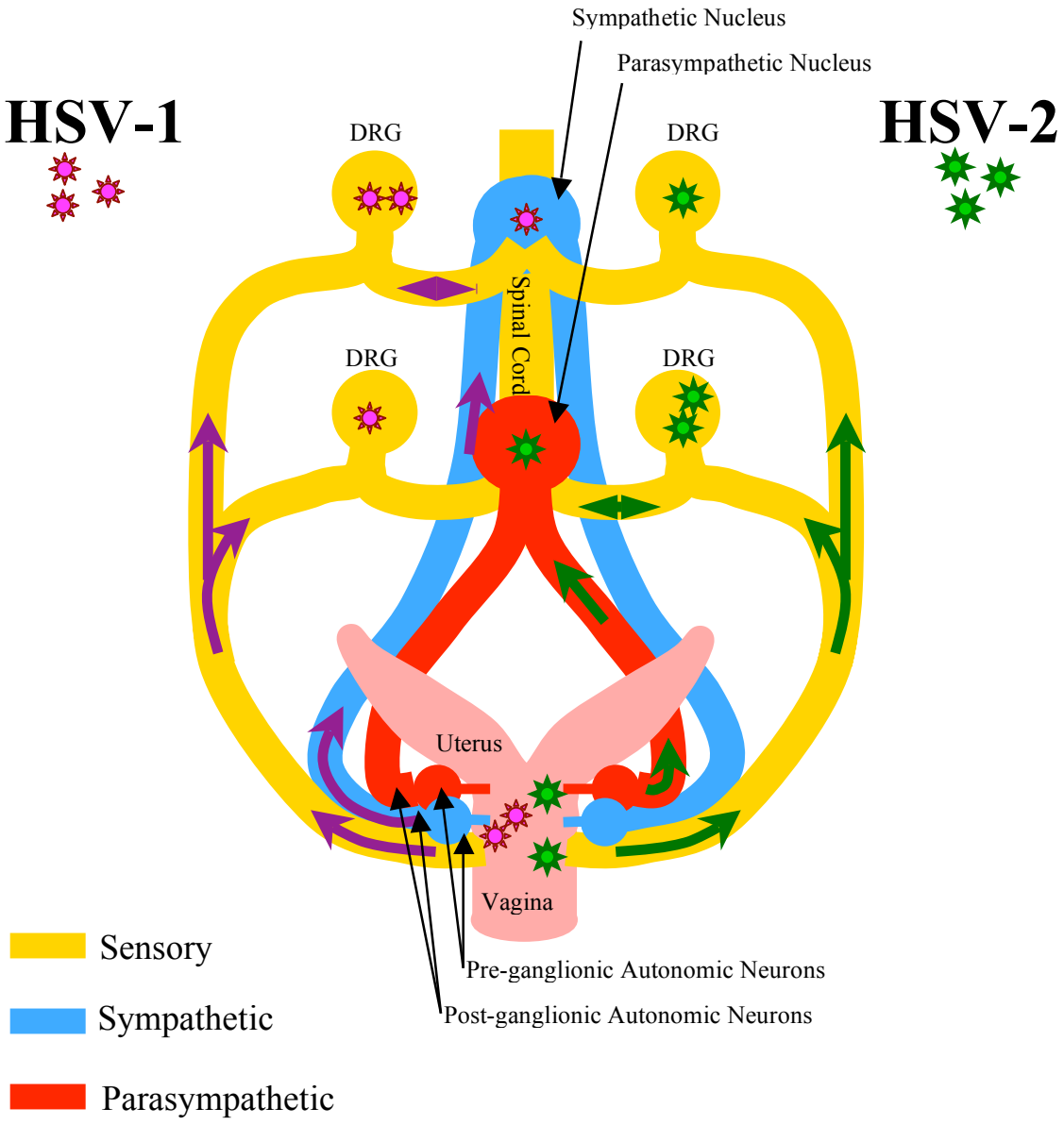
Proposed Model

Taken together, the results support some modifications to the present model of HSV latency and reactivation. Figure 38 illustrates the proposed model for HSV latency and reactivation, taking into consideration the involvement of the autonomic nervous system and the spinal cord. HSV enters the epithelium where it gains entry into free nerve endings of sensory neurons. The virus is transported by retrograde axonal flow to the sensory neuronal cell bodies that lie in the innervating ganglia, trigeminal ganglia (TG) for HSV-1 and lumbosacral dorsal root ganglia (DRG) for HSV-2, although HSV-1 is capable of establishing latency in DRG as well. In the ganglia, the virus establishes life-long latency. The virus also gains entry into nerve endings of autonomic neurons in the epithelium and is transported by retrograde axonal flow to the autonomic ganglia where the virus crosses the synapses into pre-ganglionic autonomic neuron axons. The virus can then transport retrogradely to the sympathetic nucleus in the lumbar region of the spinal cord or the parasympathetic nucleus in the sacral spinal cord (after genital infection). HSV-1 preferentially spreads via the sympathetic pathway while HSV-2 prefers the parasympathetic, selectively reaching either the lumbar cord or the sacral cord, respectively. In the autonomic nuclei, the virus replicates and enters the central branch of the sensory neurons, and is transported by retrograde axonal flow to the sensory neuronal cell body in the DRG. From the spinal cord, virus selectively enters synaptically connected sensory neurons that support latency rather than the productive cycle upon entry. In response to various stimuli, such as stress, fever, or UV light, the virus may periodically reactivate from either the sensory neurons in the DRG or the autonomic neurons in the spinal cord to cause recurrent disease at or near the original site

of inoculation. The specific mechanisms for reactivation, and particularly type-specific reactivation, are not understood but the latency-associated transcript plays a distinctive role. The LAT sequence influences the establishment of latency and viral spread, suggesting different virus-host interactions in different types of neurons. A likely mechanism by which the LAT region sequences could exert cell-type specific effects is through LAT enhancer elements that are required for efficient establishment of or reactivation from latency. The cAMP response element in the LAT promoter in concert with an unidentified element (possibly an AP-2α transcription factor binding site) near the PvuI restriction site just downstream of the TATA box determine the expression of productive or latent transcripts dependent on specific cellular factors in different types of neurons, which determines the establishment of latency in neurons that are susceptible to reactivation in response to specific stimuli. HSV-1 and HSV-2 establish latency in different subtypes of neurons that presumably respond to appropriate stimuli that selectively activate signaling cascades that reactivate the viruses to provide suitable opportunities for transmission. NFkB binding sites present in LAT exon 1 may also aid in restricting reactivation of HSV-1, possibly by increasing a stimulus threshold that the virus must overcome to reactivate. HSV-1 and HSV-2 spread through the central nervous system as a natural course of viral entry. In the CNS, the viruses typically maintain a well-regulated balance between replication and latency. However, stressors and immunological factors likely contribute to a disruption of viral life cycle regulation that can result in CNS disease, typically encephalitis for HSV-1 and meningitis for HSV-2 due to the neuronal pathways preferentially employed for each virus.

Figure 38. Proposed Model for HSV Latency in Lumbosacral DRG.

HSV-1 (purple stars and arrows) and HSV-2 (green stars and arrows) enter the epithelium where they gain entry into free nerve endings of sensory neurons (yellow). The viruses are transported by retrograde axonal flow to the DRG, where they establish latency. The viruses also appear to enter autonomic nerve endings in the epithelium. HSV-1 preferentially spreads via the sympathetic pathway (blue) while HSV-2 preferentially spreads via the parasympathetic pathway (red). The viruses cross the synapses from pre-ganglionic into post-ganglionic autonomic neurons and are transported by retrograde axonal flow to the sympathetic nucleus in the lumbar cord, more likely for HSV-1, or the parasympathetic nucleus in the sacral cord, more likely for HSV-2. In the autonomic nuclei in the spinal cord, the virus replicates and enters the central branch of the sensory neurons, selectively targeted to synaptically connected sensory neurons that support latency rather than the productive cycle. In response to various stimuli, the viruses may periodically reactivate from either sensory neurons in the DRG or autonomic neurons in the spinal cord or paracervical ganglia to cause recurrent disease at or near the original site of inoculation.


HSV-1
HSV-2
Sympathetic Nucleus
Parasympathetic Nucleus
DRG
DRG
DRG
DRG
Spinal Cord
Uterus
Vagina
Sensory
Sympathetic
Parasympathetic
Pre-ganglionic Autonomic Neurons
Post-ganglionic Autonomic Neurons

Biological Significance of These Discoveries

In the current paradigm of HSV latency, the virus gains access to the sensory neurons and is transported in a retrograde fashion to the innervating sensory ganglia, where the virus establishes a latent infection from which it can periodically reactivate in response to appropriate stimuli. HSV is thought to rarely enter the central nervous system, and is assumed to cause symptomatic disease when the virus does reach the CNS. The results presented here demonstrate that HSV is not limited to sensory neurons but also gains access to the central nervous system and autonomic neurons as a normal course of infection, and the virus establishes latency in neurons within the spinal cord, which may be post-ganglionic autonomic neurons or interneurons that are synaptically connected to sensory and autonomic neurons. In the guinea pig model, the autonomic nervous system and the spinal cord play an integral role in viral spread and the severity of disease, and reactivation appears to occur within the neurons of the spinal cord in addition to those within the DRG. The guinea pig genital model closely mimics human HSV infection, following a disease course with asymptomatic or symptomatic acute infection characterized by vesicular lesions on an erythematous base followed by establishment of latency and periodic spontaneous recurrences. The guinea pigs do not characteristically develop encephalitis with wild type virus, although some do demonstrate symptoms of radiculopathy and autonomic nervous system dysfunction during acute infection, which is also consistent with human epidemiological data.

The findings in the guinea pig model suggest that type-specific differences in reactivation and CNS disease between HSV-1 and HSV-2 may be closely related to their preferred sites of latency and replication in the central and autonomic nervous systems, in

addition to their preference for specific types of neurons in the DRG, which has implications for the pathogenesis of peripheral and CNS disease in humans and potential alternative treatments. HSV-2 invades both the sacral and lumbar regions of the spinal cord in the guinea pig, but efficiently expresses viral transcripts only in the sacral region of the cord, suggesting that replication in the autonomic nervous system is limited to the parasympathetic neurons. HSV-2 replication is thus restricted to the sacral spinal cord and CNS involvement is generally limited to neurons in the sacral region of the cord, resulting in sacral meningitis and radiculomyelopathy. More severe disease is associated with increased involvement of the autonomic nervous system and increased replication in neurons of the spinal cord.

Since neuronal stimulation has been implicated in increased viral reactivation and replication, pharmacological agents that inhibit autonomic neurons may prove useful in the control of viral replication within autonomic neurons, potentially reducing the frequency of viral reactivation and the severity of HSV-associated CNS disease. Atropine, tolterodine (Detrol^{TM}) and oxybutynin (Ditropan^{TM}) are anticholinergic agents that block cholinergic receptors or release of acetylcholine, inhibiting stimulation of autonomic neurons, which may inhibit reactivation and replication of HSV in those neurons. Beta blockers may also be potentially useful as pharmacological agents against HSV reactivation and replication by inhibiting sympathetic neurons.

Data presented here suggest opportunities for the development of targeted antivirals designed to exploit specific mechanisms of latency reactivation. The critical regions of the LAT contain a series of regulatory elements that function cooperatively to regulate transcription of either latent or productive cycle genes. These functional

elements are dependent on specific unidentified neuronal factors that permit latency or replication within specific neurons, which is the determining factor in type-specific differences in reactivation and CNS manifestations of HSV-1 and HSV-2. The work presented here provides insight into potential mechanisms by which the LAT region may regulate these type-specific differences, including putative transcription factor binding sites for AP2α and NFkB in LAT exon 1 as well as a cAMP response element in the LAT promoter. Although additional studies are needed to demonstrate the function of these sites and determine the precise mechanism of action, targeted antivirals that selectively inhibit the mechanism of reactivation from latency could be potentially beneficial if the virus could be "locked" in a latent state, preventing reactivation into an actively replicating virus capable of causing peripheral or central nervous system disease. One potential candidate would be peptide-conjugated phosphorodiamidate morpholino oligomers (P-PMO), which function by sequence-specific steric blockage of transcription and have successfully inhibited RNA viruses in cell culture and prevented lethal encephalitis from Venezuelan equine encephalitis virus in mice (add reference at home). P-PMO directed toward LAT exon 1 could potentially inhibit recurrences by sterically blocking the binding of neuronal factors required for reactivation. Alternatively, peptide "locks" could be designed to antagonize specific interactions between neuronal factors and viral regulatory elements or irreversibly bind to the regulatory elements to prevent virus-host factor interactions.

The work presented here provides the groundwork for additional studies on the pathogenesis of the virus and the mechanisms governing the establishment of latency and reactivation. HSV is not limited to the DRG during the normal course of infection and

additional studies are needed to evaluate the interactions between specific neuronal factors and the virus within neurons in the central and autonomic nervous systems in addition to those in the DRG. To fully understand the pathogenesis of HSV, studies must focus on the neuronal environment in which the virus resides and the specific cellular events and signaling cascades that trigger latent or productive cycle transcription, in autonomic neurons and interneurons in addition to sensory neurons.

Chapter 7

Supplementary Data

GFP-Expressing Viruses

HSV latency and reactivation is notoriously difficult to study due to the necessity for animal studies to evaluate the biologically relevant mechanisms. Many aspects of HSV-1 pathogenesis can be evaluated in mice or rabbits. However, HSV-2 is more virulent than HSV-1 in mice and rabbits and requires administration of acyclovir, which alters replication and establishment of latency. The accepted model for latency and reactivation studies for HSV-2 is the guinea pig. Since all guinea pig strains are outbred, variability is a distinct disadvantage with this model and typically necessitates larger numbers of animals to obtain statistically significant differences. HSV infection in each individual animal progresses differently so a method of studying viral spread and reactivation in a single animal would be extremely advantageous.

In vivo imaging provides an opportunity to observe the progression of replicating virus through an individual animal. To enable visualization of replicating virus, green fluorescent protein (GFP)-expressing HSV-1 and HSV-2 viruses were constructed and characterized *in vitro*. The biological activity of the GFP-expressing viruses was verified in guinea pigs, although technical limitations prevented further *in vivo* characterization of these viruses.

The HSV viral capsid is decorated with a small protein VP26, outside the primary capsid proteins but within the tegument and envelope (Figure 39). The GFP-expressing viruses that were constructed produce a GFP-VP26 fusion protein, which effectively

decorates each viral capsid with an estimated 200-300 molecules of GFP. Once attached to the viral capsid assembly, the VP26-GFP fusion protein is protected inside the envelope. The GFP-expressing viruses are readily visualized within cells (Figure 41).

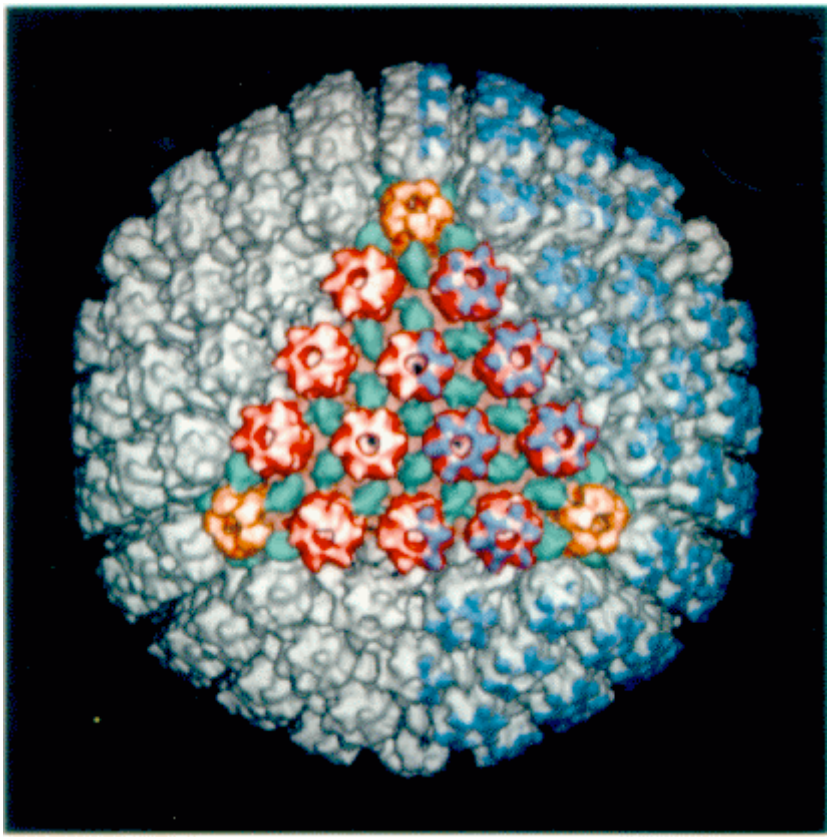


Figure 39. HSV Capsid.

The virus capsid is made up of VP5, VP19, VP23, and VP26. VP5 makes pentons (orange) and hexons (red), connected by triplexes of VP19 and VP23 (green). Hexons are decorated at the distal tips by VP26 (blue). GFP viruses express VP26-GFP fusion protein which decorates the virus capsid during replication and assembly. There are an estimated 300-600 VP26-GFP copies decorating the capsid, making the virus highly visible (Jay C. Brown, Univ. of Virginia, Dept. of Microbiology and Cancer Center, Herpes Virus Research Lab).

HSV-1 and HSV-2 GFP-Expressing Virus Construction.

For viruses expressing green fluorescent protein (GFP), a previously described plasmid construct designated pK26GFP (21) was generously provided by Dr. Prashant Desai (Johns Hopkins University, Baltimore, MD). The construct contained GFP inserted after the third amino acid in the HSV-1 viral protein VP26 (Figure 40). During the productive cycle, the virus expresses the VP26-GFP fusion protein, which then decorates the viral capsid. This plasmid was used with parent DNA HSV-1 strain 17+ and HSV-2 strain 333 to construct 17+GFP (HSV-1) and 333-GFP (HSV-2) by homologous recombination as described above. Sequencing of the VP26 gene and flanking sequences verified correct insertion of the GFP DNA sequence. Although pK26GFP was constructed from HSV-1 VP26, sequencing of 333-GFP demonstrated that homologous recombination occurred in a region of identity immediately downstream of GFP and the GFP-expressing virus contained only HSV-2 VP26 sequences.

To compare growth characteristics of the GFP-expressing viruses with wild type HSV-1 and HSV-2 in cell culture, ~10^6 Vero cells were infected with each virus at an MOI of 0.1. Cells were harvested at 0, 2, 5, 12, and 20 hours post-infection, scraped, freeze-thawed three times, and plaque-titered in duplicate. 17+GFP had one-step growth kinetics similar to the wild type HSV-1 strain 17+ (Figure 42). Infection with 333-GFP resulted in a 10-fold lower viral titer compared to wild type HSV-2 strain 333. 333-GFP was re-constructed three separate times and each time, the GFP-expressing HSV-2 had similar growth kinetics.

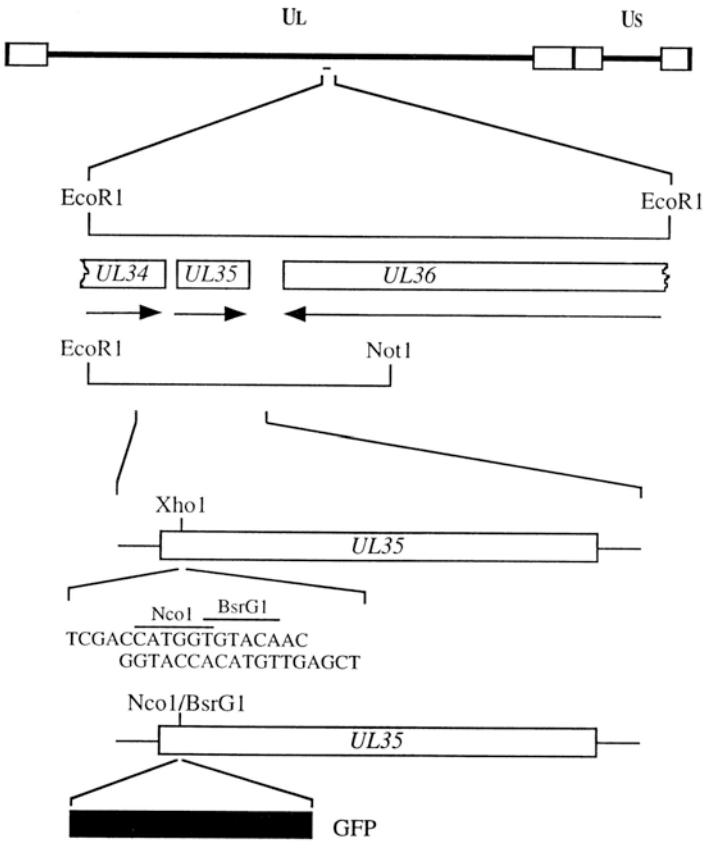


Figure 40. Construction of GFP-Expressing Viruses 17+GFP and 333-GFP.

The 5.2-kb *Eco*RI L fragment of HSV-1 strain KOS was cloned into pUC19. The *Eco*RI L fragment contains UL35 (VP26) and the C-terminal-encoding sequences of the UL34 and UL36 genes. A shortened version of the *Eco*RI L clone spanning the *Eco*RI and *NotI* restriction sites was used for subsequent manipulations. An *Xho*I restriction site that spans residues 5 and 7 of the UL35 ORF was created by overlap extension PCR assays. This plasmid was cleaved with *Xho*I, and an oligonucleotide duplex specifying *Nco*I and *Bsr*GI restriction sites was annealed at this position. The GFP ORF derived from pEGFP-N1 (Clontech) as an *Nco*I-*Bsr*GI fragment was cloned into this plasmid to generate pK26GFP. U_L, unique long; U_S, unique short. (21)

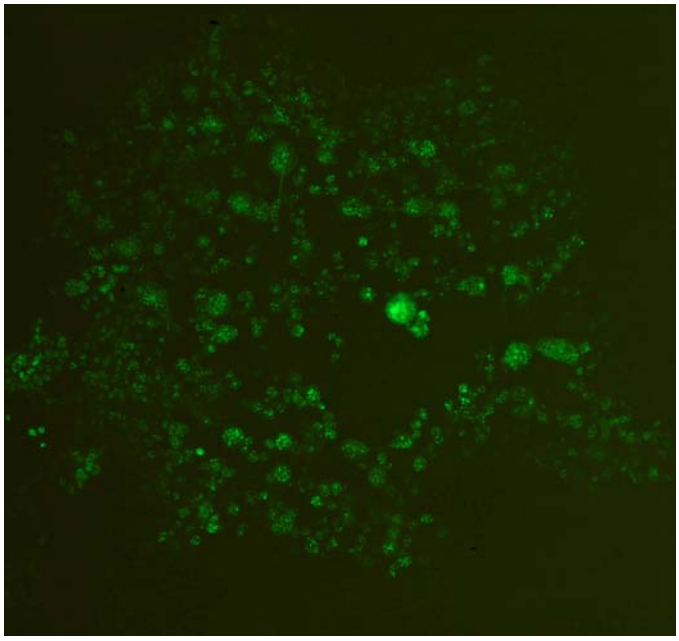


Figure 41. 17+GFP viral plaque.

Characteristic viral plaque with clearing in the center, surrounded by rounded virus-infected cells expressing GFP. 17+GFP and 333-GFP viral plaques were indistinguishable.

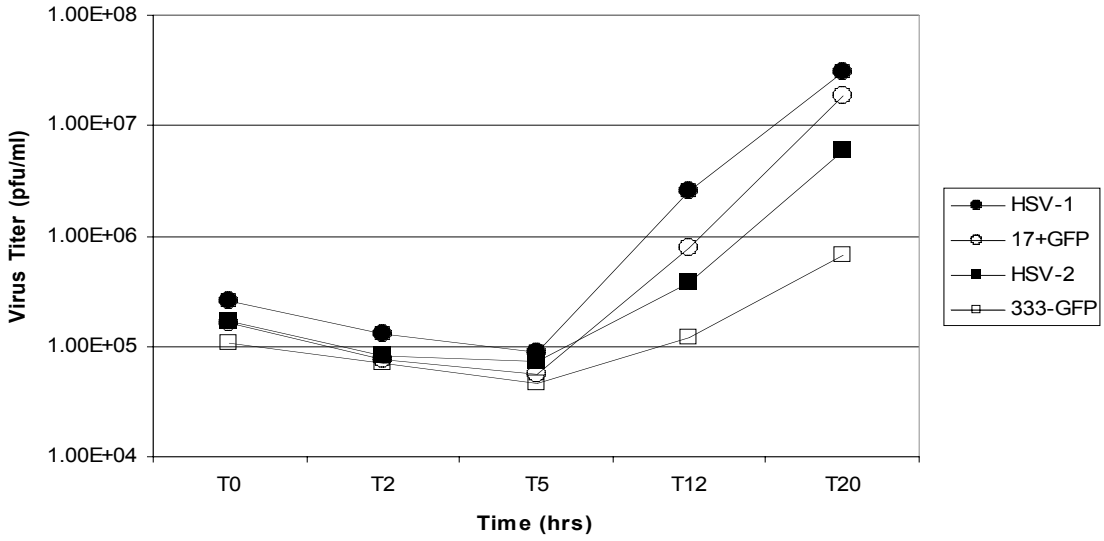


Figure 42. One-Step Growth Curves - GFP Viruses

To compare one-step growth characeristics of wild type and GFP-expressing viruses, ~106 Vero cells were inouclatioed in duplicate at time 0 with a mutltiplicity of infectionof approximately 0.1 pfu/cell of each virus. Medium was added after a two-hour adsorption period. At 0, 2, 5, 12, and 20 hours post-infection, cells were scraped, freeze-thawed three time, and plaque-titered in duplicate. 17+GFP is HSV-1 strain 17+ and 333-GFP is HSV-2 strain 333. *17+GFP replicates in cell culture similar to wild type HSV-1, but 333-GFP replicates with slower kinetics in cell culture compared to wild type HSV-2.*

Results

GFP-expressing HSV-1 and HSV-2 produce similar acute lesion severity.

To determine if the GFP-expressing viruses were biologically equivalent to wild type HSV-1 and HSV-2 viruses, 17+GFP and 333-GFP were evaluated in the guinea pig genital model. Female guinea pigs were inoculated intravaginally with 10^5 pfu of 17+GFP or 333-GFP. The severity of lesions was compared during the acute phase of infection through Day 14 post-inoculation (Figure 43). The mean lesion scores were similar between 17+GFP and 333-GFP, although both produced acute infections that were less severe than typically observed with wild type viruses. Wild type HSV-1 and HSV-2 typically reach a mean peak lesion severity near 3.5 on the 4-point severity scale, while the GFP-expressing viruses peaked at a mean of near 2 in this preliminary evaluation. A reduction in acute severity was expected for 333-GFP, given its growth characteristics in cell culture, so the observation of acute symptoms similar in character to that produced by 17+GFP was encouraging. Although VP26 may not be critical for productive infection (21), the protein apparently is of some importance to the viral life cycle.

333-GFP reactivates more efficiently than 17+GFP.

During latent infection, 333-GFP reactivated more efficiently than 17+GFP after genital inoculation, even though the acute infections were less severe than typically observed during wild type infection (Figure 44). These results suggest that the 333-GFP and 17+GFP are biologically functional and reactivate with relative kinetics comparable

to wild type HSV-2 and HSV-1. The GFP-expressing viruses provide a functional tool for visual observation of differences in viral spread and reactivation.

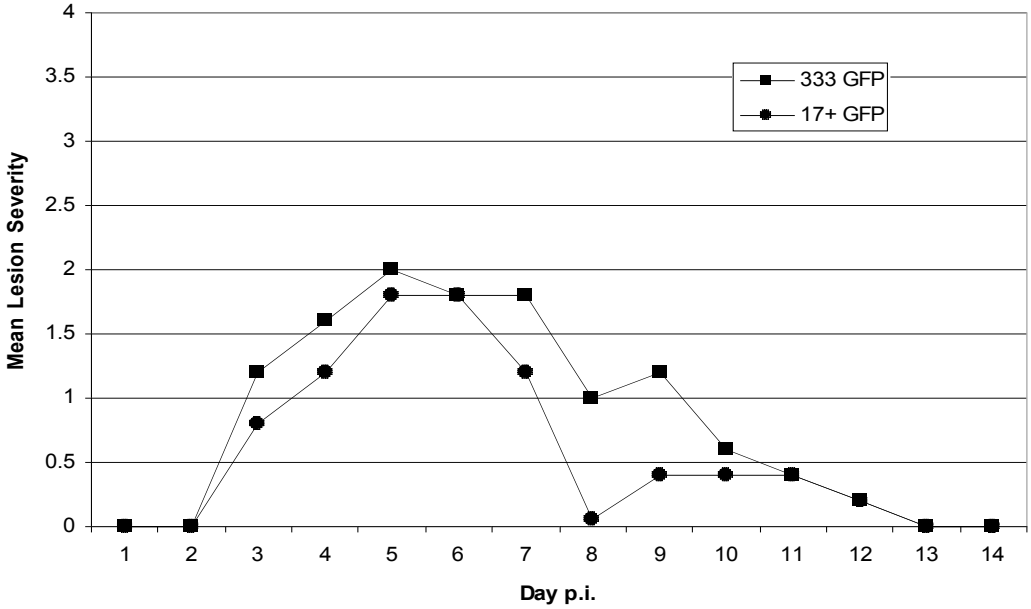


Figure 43. Acute Lesion Severity - 333-GFP and 17+GFP.

Lesion severity is graphed as the mean for each group of guinea pigs oneach day of observation from Days 1-14 p.i., with 0 being no symptoms and 4 being the most severe. 333-GFP (n=5), 17+GFP (n=5). *Acute lesion severity was similar between 17+GFP and 333-GFP, even though 333-GFP replicated with slower kinetics in cell culture.*

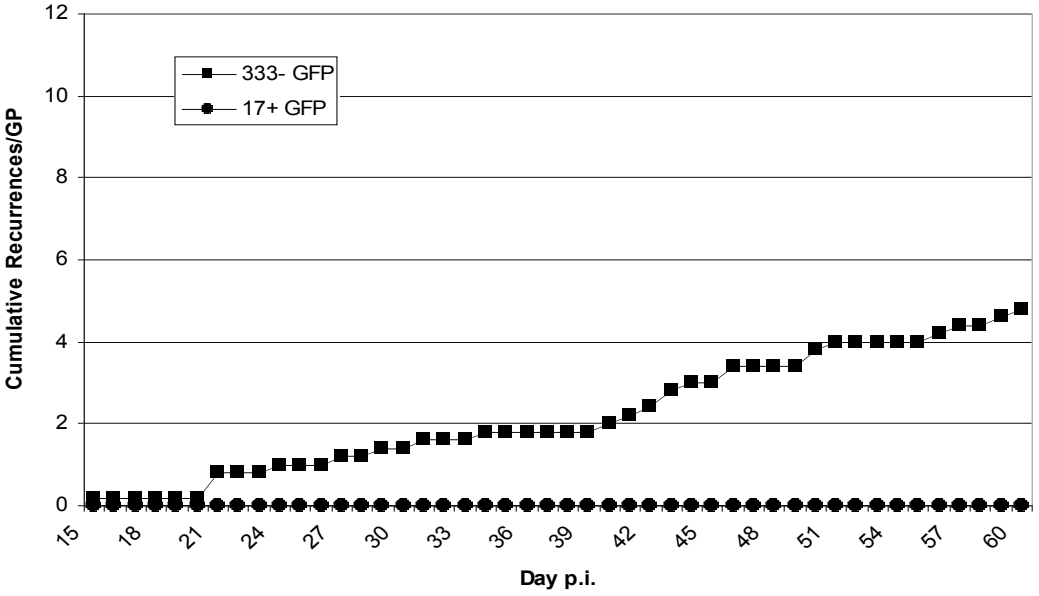


Figure 44. Cumulative recurrences from 333-GFP and 17+GFP.

Cumulative recurrences per guinea pig for each group. 333-GFP (n=5), 17+GFP (n=5). *333-GFP reactivated more efficiently than 17+GFP after genital infection, consistent with wild type virus comparisons, verifying the biological activity of the GFP-expressing viruses.*

Discussion

The GFP-expressing HSV-1 and HSV-2 viruses will be critical tools in assessing spread of the viruses and reactivation. Although technical issues prevented further characterization of 17+GFP and 333-GFP *in vivo*, these viruses will be used in future studies to visualize replicating virus in tissue sections removed from guinea pigs and also to serially image the virus in live guinea pigs.

17+GFP and 333-GFP can be utilized in several ways. Infected guinea pigs can be imaged daily to follow the spread of replicating virus through the nervous system throughout the course of infection. In this way, viral spread can be visualized in an individual animal rather than sacrificing several animals to obtain an average "snapshot" of viral spread at a given time point.

The GFP-expressing viruses can also be used as a tool for verification of viral progression prior to sacrifice at given time points. Currently, several guinea pigs are sacrificed at a given time point without clear knowledge of the extent of viral progression. The first 3-5 days after infection are critical for viral spread and the establishment of latency, since the virus has replicated in neurons and latency is already established by the time symptoms appear. Studying viral spread at these early time points is difficult and requires additional animals because one must account for those animals in which the virus fails to produce an infection. At later time points, the presence of symptoms allows the investigator to choose animals that have comparable infections for comparisons but prior to the appearance of symptoms, the investigator is blinded to the progression of infection, which introduces additional variability. With the GFP-expressing viruses, live animals can be imaged to ascertain whether the virus is

replicating prior to the appearance of external symptoms, reducing the requirement for additional animals in the study to account for the variability.

The GFP-expressing viruses will also provide a tool for visualizing replicating virus within anatomical sites that have not been sufficiently analyzed, such as the autonomic ganglia and nuclei. By expressing GFP, HSV-1 and HSV-2 can be visualized within these autonomic structures to definitely ascertain the role of the autonomic pathways in viral spread. Work presented here suggests that reactivation may occur in autonomic neurons in addition to sensory neurons. During latency, the viral genome is quiescent except for the expression of LAT so viral capsids would not be produced and GFP would not be expressed. Upon reactivation, the virus would produce GFP-decorated capsids and a recurrence could theoretically be visualized within specific neuronal cell bodies prior to the appearance of external lesions, providing insight into the mechanics of reactivation.

HSV-2 cAMP Response Element Mutant Virus (CRE)

The cAMP response element (CRE) in the promoter of HSV-1 has been identified as an inducible element critical for reactivation (11, 56, 66). The CRE in the promoter of HSV-2 has not been analyzed. An HSV-2 CRE mutant was constructed by Hiroki Takakuwa in the Krause lab by site-directed mutagenesis of two base pairs to destroy the CRE consensus sequence and generate a restriction enzyme site. By generating a restriction enzyme site, the mutant virus could be readily identified by PCR followed by restriction endonuclease digestion. The HSV-2 CRE mutant virus was purified by plaque purification and verified by Southern blot (data not shown since this portion of the work on this virus was performed by Hiroki Takakuwa). The HSV-2 CRE mutant was evaluated in both the footpad and genital guinea pigs models of infection. A rescuant of the HSV-2 CRE mutant was also constructed and evaluated in the genital model.

Results

cAMP response element influences acute infection after genital inoculation but not after footpad inoculation.

To determine the effects of mutating the cAMP response element in the promoter of HSV-2, the HSV-2 CRE mutant was evaluated in the guinea pig genital and footpad models of infection (Figure 44). After footpad inoculation, the CRE mutant produced an acute infection similar in lesion severity to HSV-2. However after genital infection, the acute lesion severity produced by the CRE mutant was significantly less than HSV-2, although the CRE rescuant also produced a less severe acute infection compared to wild type HSV-2. Since the HSV-2 CRE ablation had no effect in the footpad model, but

significantly reduced the severity of the acute infection in the genital model, these results suggest that the HSV-2 CRE may only effect disease severity when the virus has access to both sensory and autonomic pathways. Thus, the HSV-2 CRE in the LAT promoter may only be functional in autonomic neurons.

The cAMP response element is critical for efficient reactivation of HSV-2.

During latent infection, wild type HSV-2 reactivated more efficiently than the HSV-2 CRE mutant, after both footpad and genital inoculations (Figure 45). After genital inoculation, the rescuant of the CRE mutant also reactivated more efficiently than the HSV-2 CRE mutant, verifying that the reduced reactivation frequency was due to the CRE ablation in the mutant virus. The rescuant demonstrated a decrease in acute disease severity and reactivated somewhat less efficiently than wild type HSV-2, which could potentially have resulted from a reduced viral inoculum although a secondary mutation cannot be ruled out at this time. The virus titer will be verified prior to subsequent animal experiments. Additional animal experiments are required to verify the phenotypes of the mutant and rescuant viruses in comparison with wild type HSV-2.

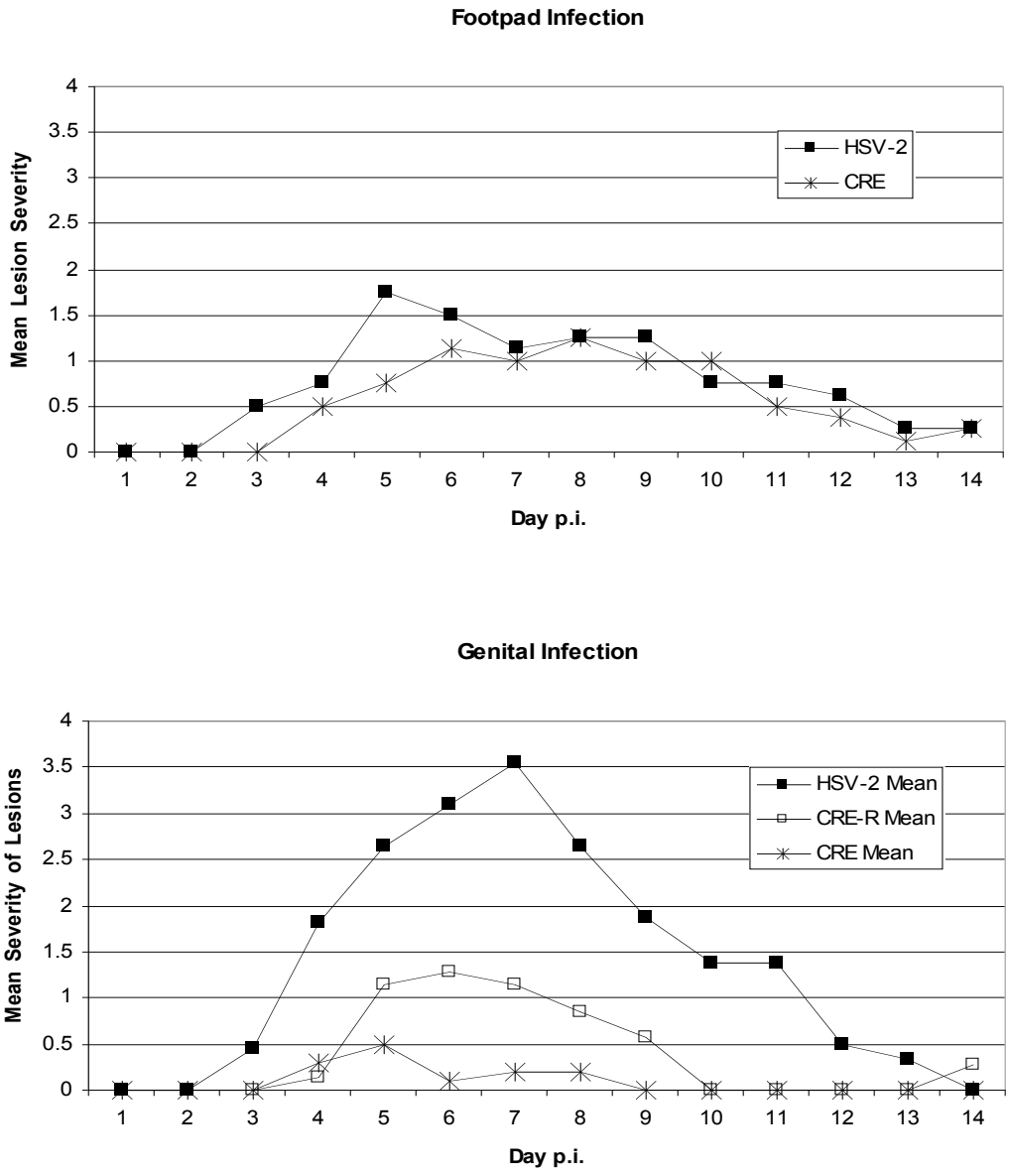


Figure 45. Acute lesion severity after footpad and genital infection of CRE mutant.

Lesion severity is graphed as the mean for each group of guinea pigs on each day of observation from Days 1-14 p.i., with 0 being no symptoms and 4 being the most severe. HSV-2 (n=8 footpad, n=8 genital), CRE (n=8 footpad, n=8 genital), CRE-R (n=8 genital). *These results suggest that the HSV-2 CRE may only effect disease severity when the virus has access to both sensory and autonomic pathways. Thus, the HSV-2 CRE in the LAT promoter may only be functional in autonomic neurons.*

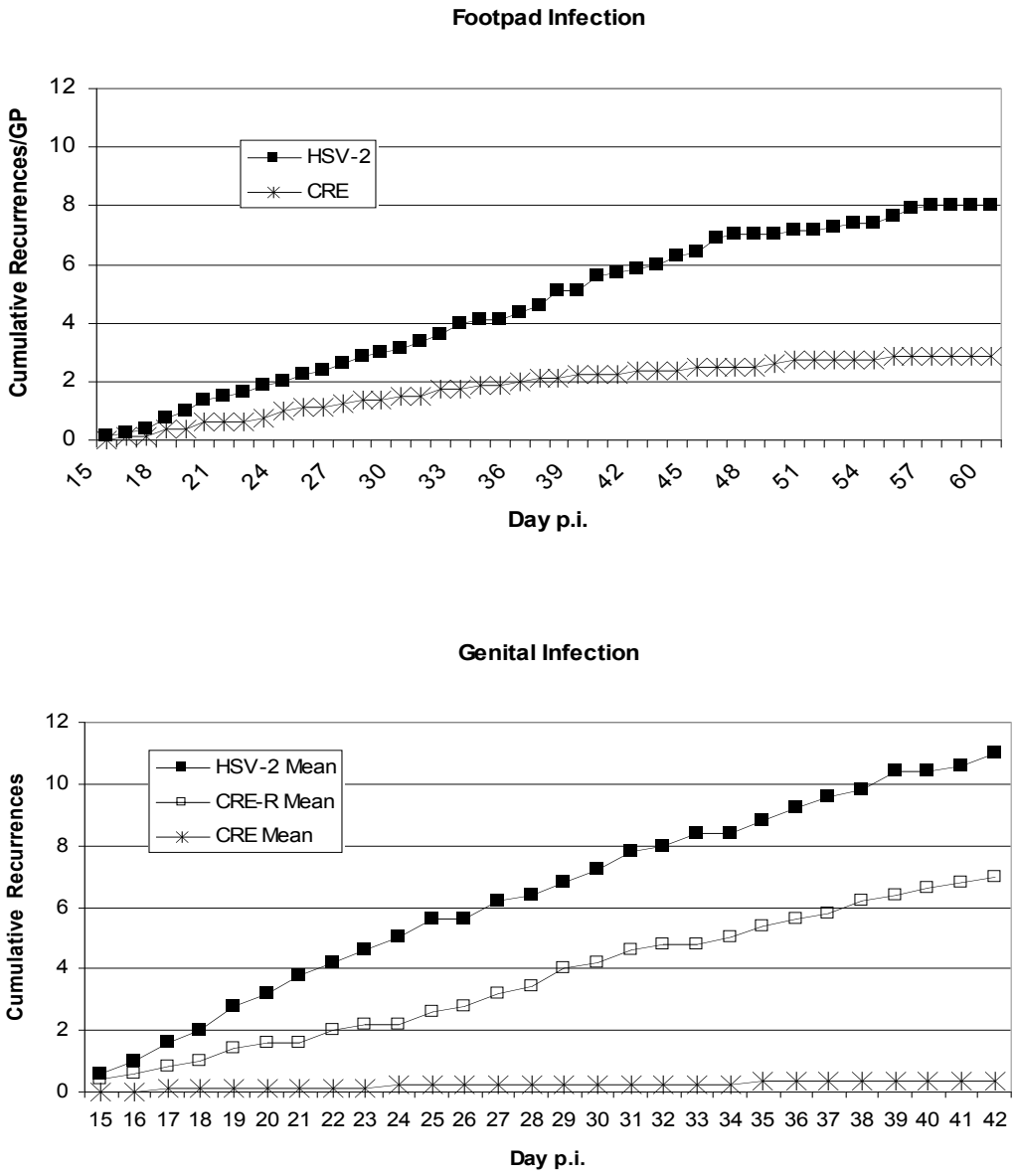


Figure 46. Cumulative recurrences from CRE mutant after footpad and genital inoculation.

Cumulative recurrences per guinea pig for each group after footpad (top graph) and genital (bottom graph) inoculations. HSV-2 (n=8 footpad, n=8 genital), CRE (n=8 footpad, n=8 genital), CRE-R (n=8 genital). *Wild type HSV-2 and the HSV-2 CRE rescuant reactivated more efficiently than the HSV-2 CRE mutant, demonstrating that the CRE in the HSV-2 LAT promoter is critical for efficient reactivation of HSV-2.*

Discussion

The HSV-2 CRE mutation resulted in less severe acute disease after genital infection but not after footpad infection, suggesting that the CRE may only affect disease severity when the virus has access to both sensory and autonomic pathways. Thus, the HSV-2 CRE in the LAT promoter may only be functional in neurons of the autonomic pathways.

The CRE mutation also dramatically reduced the recurrence frequency of the virus. Although the rescuant also demonstrated a somewhat reduced recurrence frequency, the difference between the rescuant and the wild type viruses was not statistically significant. Thus, the cAMP response element in the HSV-2 LAT promoter is critical for reactivation of HSV-2.

Additional studies are required to fully characterize the effects of the HSV-2 LAT CRE mutation, including evaluation of the distribution of latently-infected neurons in the peripheral and central nervous system and molecular analyses to determine the specific mechanism by which the CRE functions.

References

1. **Ackland-Berglund, C. E., D. J. Davido, and D. A. Leib.** 1995. The roles of the cAMP-response element and TATA box in expression of the herpes simplex virus type 1 latency-associated transcripts. Virology **210:**141-51.
2. **Ahmed, M., M. Lock, C. G. Miller, and N. W. Fraser.** 2002. Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis in vitro and protect neuronal cells in vivo. J Virol **76:**717-29.
3. **Arthur, J. L., R. Everett, I. Brierley, and S. Efstathiou.** 1998. Disruption of the 5' and 3' splice sites flanking the major latency-associated transcripts of herpes simplex virus type 1: evidence for alternate splicing in lytic and latent infections. J Gen Virol **79 (Pt 1):**107-16.
4. **Aubert, M., and J. A. Blaho.** 2001. Modulation of apoptosis during herpes simplex virus infection in human cells. Microbes Infect **3:**859-66.
5. **Berthomme, H., J. Lokensgard, L. Yang, T. Margolis, and L. T. Feldman.** 2000. Evidence for a bidirectional element located downstream from the herpes simplex virus type 1 latency-associated promoter that increases its activity during latency. J Virol **74:**3613-22.
6. **Bhattacharjee, P. S., R. K. Tran, M. E. Myles, K. Maruyama, A. Mallakin, D. C. Bloom, and J. M. Hill.** 2003. Overlapping subdeletions within a 348-bp in the 5' exon of the LAT region that facilitates epinephrine-induced reactivation of HSV-1 in the rabbit ocular model do not further define a functional element. Virology **312:**151-8.
7. **Block, T. M., S. Deshmane, J. Masonis, J. Maggioncalda, T. Valyi-Nagi, and N. W. Fraser.** 1993. An HSV LAT null mutant reactivates slowly from latent infection and makes small plaques on CV-1 monolayers. Virology **192:**618-30.
8. **Block, T. M., J. G. Spivack, I. Steiner, S. Deshmane, M. T. McIntosh, R. P. Lirette, and N. W. Fraser.** 1990. A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. J Virol **64:**3417-26.
9. **Bloom, D. C., G. B. Devi-Rao, J. M. Hill, J. G. Stevens, and E. K. Wagner.** 1994. Molecular analysis of herpes simplex virus type 1

during epinephrine-induced reactivation of latently infected rabbits in vivo. J Virol **68:**1283-92.

10. **Bloom, D. C., J. M. Hill, G. Devi-Rao, E. K. Wagner, L. T. Feldman, and J. G. Stevens.** 1996. A 348-base-pair region in the latency-associated transcript facilitates herpes simplex virus type 1 reactivation. J Virol **70:**2449-59.
11. **Bloom, D. C., J. G. Stevens, J. M. Hill, and R. K. Tran.** 1997. Mutagenesis of a cAMP response element within the latency-associated transcript promoter of HSV-1 reduces adrenergic reactivation. Virology **236:**202-7.
12. **Bourne, N., L. R. Stanberry, B. L. Connelly, J. Kurawadwala, S. E. Straus, and P. R. Krause.** 1994. Quantity of latency-associated transcript produced by herpes simplex virus is not predictive of the frequency of experimental recurrent genital herpes. J Infect Dis **169:**1084-7.
13. **Branco, F. J., and N. W. Fraser.** 2005. Herpes simplex virus type 1 latency-associated transcript expression protects trigeminal ganglion neurons from apoptosis. J Virol **79:**9019-25.
14. **Burton, E. A., C. S. Hong, and J. C. Glorioso.** 2003. The stable 2.0-kilobase intron of the herpes simplex virus type 1 latency-associated transcript does not function as an antisense repressor of ICP0 in nonneuronal cells. J Virol **77:**3516-30.
15. **Bustos, D. E., and S. S. Atherton.** 2002. Detection of herpes simplex virus type 1 in human ciliary ganglia. Invest Ophthalmol Vis Sci **43:**2244-9.
16. **Chu, K., D. W. Kang, J. J. Lee, and B. W. Yoon.** 2002. Atypical brainstem encephalitits caused by herpes simplex virus 2. Arch Neurol **59:**460-463.
17. **Cook, S. D., M. J. Paveloff, J. J. Doucet, A. J. Cottingham, F. Sedarati, and J. M. Hill.** 1991. Ocular herpes simplex virus reactivation in mice latently infected with latency-associated transcript mutants. Invest Ophthalmol Vis Sci **32:**1558-61.
18. **Corey, L., H. G. Adams, Z. A. Brown, and K. K. Holmes.** 1983. Genital herpes simplex virus infections: clinical manifestations, course, and complications. Ann Intern Med **98:**958-72.
19. **Corey, L., A. Wald, C. L. Celum, and T. C. Quinn.** 2004. The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: a review of two overlapping epidemics. J Acquir Immune Defic Syndr **35:**435-445.

20. **Danaher, R. J., A. D. Savells-Arb, S. A. Black, Jr., R. J. Jacob, and C. S. Miller.** 2001. Herpesvirus quiescence in neuronal cells IV: virus activation induced by pituitary adenylate cyclase-activating polypeptide (PACAP) involves the protein kinase A pathway. J Neurovirol **7:**163-8.
21. **Desai, P., and S. Person.** 1998. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. J Virol **72:**7563-8.
22. **Devi-Rao, G. B., J. S. Aguilar, M. K. Rice, H. H. Garza, Jr., D. C. Bloom, J. M. Hill, and E. K. Wagner.** 1997. Herpes simplex virus genome replication and transcription during induced reactivation in the rabbit eye. J Virol **71:**7039-47.
23. **Devi-Rao, G. B., D. C. Bloom, J. G. Stevens, and E. K. Wagner.** 1994. Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latently infected murine sensory ganglia. J Virol **68:**1271-82.
24. **Dobson, A. T., T. P. Margolis, W. A. Gomes, and L. T. Feldman.** 1995. In vivo deletion analysis of the herpes simplex virus type 1 latency-associated transcript promoter. J Virol **69:**2264-70.
25. **Dobson, A. T., F. Sederati, G. Devi-Rao, W. M. Flanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman.** 1989. Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. J Virol **63:**3844-51.
26. **Dodd, J., and T. M. Jessell.** 1985. Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. J Neurosci **5:**3278-94.
27. **Dolan, A., F. E. Jamieson, C. Cunningham, B. C. Barnett, and D. J. McGeoch.** 1998. The genome sequence of herpes simplex virus type 2. J Virol **72:**2010-21.
28. **Drolet, B. S., G. C. Perng, J. Cohen, S. M. Slanina, A. Yukht, A. B. Nesburn, and S. L. Wechsler.** 1998. The region of the herpes simplex virus type 1 LAT gene involved in spontaneous reactivation does not encode a functional protein. Virology **242:**221-32.
29. **Drolet, B. S., G. C. Perng, R. J. Villosis, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1999. Expression of the first 811 nucleotides of the herpes simplex virus type 1 latency-associated transcript (LAT) partially restores wild-type spontaneous reactivation to a LAT-null mutant. Virology **253:**96-106.

30. **Fareed, M. U., and J. G. Spivack.** 1994. Two open reading frames (ORF1 and ORF2) within the 2.0-kilobase latency-associated transcript of herpes simplex virus type 1 are not essential for reactivation from latency. J Virol **68:**8071-81.
31. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc Natl Acad Sci U S A **88:**790-4.
32. **Farrell, M. J., J. M. Hill, T. P. Margolis, J. G. Stevens, E. K. Wagner, and L. T. Feldman.** 1993. The herpes simplex virus type 1 reactivation function lies outside the latency-associated transcript open reading frame ORF-2. J Virol **67:**3653-5.
33. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J Virol **71:**5885-93.
34. **Gilden, D. H., R. Gesser, J. Smith, M. Wellish, J. J. Laguardia, R. J. Cohrs, and R. Mahalingam.** 2001. Presence of VZV and HSV-1 DNA in human nodose and celiac ganglia. Virus Genes **23:**145-7.
35. **Grabe, N.** 2002. AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol **2:**S1-15.
36. **Gupta, A., J. J. Gartner, P. Sethupathy, A. G. Hatzigeorgiou, and N. W. Fraser.** 2006. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. Nature **442:**82-5.
37. **Henderson, G., W. Peng, L. Jin, G. C. Perng, A. B. Nesburn, S. L. Wechsler, and C. Jones.** 2002. Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus type 1 latency-associated transcript. J Neurovirol **8 Suppl 2:**103-11.
38. **Hill, J., A. Patel, P. Bhattacharjee, and P. Krause.** 2003. An HSV-1 chimeric containing HSV-2 latency associated transcript (LAT) sequences has significantly reduced adrenergic reactivation in the rabbit eye model. Curr Eye Res **26:**219-24.
39. **Hill, J. M., H. H. Garza, Jr., Y. H. Su, R. Meegalla, L. A. Hanna, J. M. Loutsch, H. W. Thompson, E. D. Varnell, D. C. Bloom, and T. M. Block.** 1997. A 437-base-pair deletion at the beginning of the latency-associated transcript promoter significantly reduced adrenergically induced herpes simplex virus type 1 ocular reactivation in latently infected rabbits. J Virol **71:**6555-9.
40. **Hill, J. M., J. B. Maggioncalda, H. H. Garza, Jr., Y. H. Su, N. W. Fraser, and T. M. Block.** 1996. In vivo epinephrine reactivation of ocular herpes simplex virus type 1 in the rabbit is correlated to a 370-

base-pair region located between the promoter and the 5' end of the 2.0 kilobase latency-associated transcript. J Virol **70:**7270-4.

41. **Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology **174:**117-25.
42. **Hulsebosch, C. E., and R. E. Coggeshall.** 1982. An analysis of the axon populations in the nerves to the pelvic viscera in the rat. J Comp Neurol **211:**1-10.
43. **Izumi, K. M., A. M. McKelvey, G. Devi-Rao, E. K. Wagner, and J. G. Stevens.** 1989. Molecular and biological characterization of a type 1 herpes simplex virus (HSV-1) specifically deleted for expression of the latency-associated transcript (LAT). Microb Pathog **7:**121-34.
44. **Jarman, R. G., J. M. Loutsch, G. B. Devi-Rao, M. E. Marquart, M. P. Banaszak, X. Zheng, J. M. Hill, E. K. Wagner, and D. C. Bloom.** 2002. The region of the HSV-1 latency-associated transcript required for epinephrine-induced reactivation in the rabbit does not include the 2.0-kb intron. Virology **292:**59-69.
45. **Jin, L., W. Peng, G. C. Perng, D. J. Brick, A. B. Nesburn, C. Jones, and S. L. Wechsler.** 2003. Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. J Virol **77:**6556-61.
46. **Kennedy, P. G. E., and A. Chaudhuri.** 2002. Herpes simplex encephalitis. J Neurol Neurosurg Psychiatry.
47. **Kleinschmidt-DeMasters, B. K., and D. H. Gilden.** 2001. The expanding spectrum of herpesvirus infections of the nervous system. Brain Pathol **11:**440-51.
48. **Krause, P. R., K. D. Croen, J. M. Ostrove, and S. E. Straus.** 1990. Structural and kinetic analyses of herpes simplex virus type 1 latency-associated transcripts in human trigeminal ganglia and in cell culture. J Clin Invest **86:**235-41.
49. **Krause, P. R., L. R. Stanberry, N. Bourne, B. Connelly, J. F. Kurawadwala, A. Patel, and S. E. Straus.** 1995. Expression of the herpes simplex virus type 2 latency-associated transcript enhances spontaneous reactivation of genital herpes in latently infected guinea pigs. J Exp Med **181:**297-306.
50. **Kubat, N. J., A. L. Amelio, N. V. Giordani, and D. C. Bloom.** 2004. The herpes simplex virus type 1 latency-associated transcript (LAT) enhancer/rcr is hyperacetylated during latency independently of LAT transcription. J Virol **78:**12508-18.

51. **Labetoulle, M., P. Kucera, G. Ugolini, F. Lafay, E. Frau, H. Offret, and A. Flamand.** 2000. Neuronal pathways for the propagation of herpes simplex virus type 1 from one retina to the other in a murine model. J Gen Virol **81:**1201-10.
52. **Labetoulle, M., P. Kucera, G. Ugolini, F. Lafay, E. Frau, H. Offret, and A. Flamand.** 2000. Neuronal propagation of HSV1 from the oral mucosa to the eye. Invest Ophthalmol Vis Sci **41:**2600-6.
53. **Lafferty, W. E., R. W. Coombs, J. Benedetti, C. Critchlow, and L. Corey.** 1987. Recurrences after oral and genital herpes simplex virus infection. Influence of site of infection and viral type. N Engl J Med **316:**1444-9.
54. **LaVail, J. H., W. E. Johnson, and L. C. Spencer.** 1993. Immunohistochemical identification of trigeminal ganglion neurons that innervate the mouse cornea: relevance to intercellular spread of herpes simplex virus. J Comp Neurol **327:**133-40.
55. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J Virol **63:**2893-900.
56. **Leib, D. A., K. C. Nadeau, S. A. Rundle, and P. A. Schaffer.** 1991. The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc Natl Acad Sci U S A **88:**48-52.
57. **Lokensgard, J. R., H. Berthomme, and L. T. Feldman.** 1997. The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency. J Virol **71:**6714-9.
58. **Lokensgard, J. R., D. C. Bloom, A. T. Dobson, and L. T. Feldman.** 1994. Long-term promoter activity during herpes simplex virus latency. J Virol **68:**7148-58.
59. **Loutsch, J. M., G. C. Perng, J. M. Hill, X. Zheng, M. E. Marquart, T. M. Block, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. Identical 371-base-pair deletion mutations in the LAT genes of herpes simplex virus type 1 McKrae and 17syn+ result in different in vivo reactivation phenotypes. J Virol **73:**767-71.
60. **Mador, N., E. Braun, H. Haim, I. Ariel, A. Panet, and I. Steiner.** 2003. Transgenic mouse with the herpes simplex virus type 1 latency-

associated gene: expression and function of the transgene. J Virol **77:**12421-9.

61. **Mador, N., D. Goldenberg, O. Cohen, A. Panet, and I. Steiner.** 1998. Herpes simplex virus type 1 latency-associated transcripts suppress viral replication and reduce immediate-early gene mRNA levels in a neuronal cell line. J Virol **72:**5067-75.
62. **Maggioncalda, J., A. Mehta, N. W. Fraser, and T. M. Block.** 1994. Analysis of a herpes simplex virus type 1 LAT mutant with a deletion between the putative promoter and the 5' end of the 2.0-kilobase transcript. J Virol **68:**7816-24.
63. **Maggioncalda, J., A. Mehta, Y. H. Su, N. W. Fraser, and T. M. Block.** 1996. Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia. Virology **225:**72-81.
64. **Margolis, T. P., D. C. Bloom, A. T. Dobson, L. T. Feldman, and J. G. Stevens.** 1993. Decreased reporter gene expression during latent infection with HSV LAT promoter constructs. Virology **197:**585-92.
65. **Margolis, T. P., Y. Imai, L. Yang, V. Vallas, and P. R. Krause.** 2007. Herpes Simplex Virus Type 2 (HSV-2) Establishes Latent Infection in a Different Population of Ganglionic Neurons than HSV-1: Role of Latency-Associated Transcripts. J Virol **81:**1872-8.
66. **Marquart, M. E., X. Zheng, R. K. Tran, H. W. Thompson, D. C. Bloom, and J. M. Hill.** 2001. A cAMP response element within the latency-associated transcript promoter of HSV-1 facilitates induced ocular reactivation in a mouse hyperthermia model. Virology **284:**62-9.
67. **Mathews, D. H., J. Sabina, M. Zuker, and D. H. Turner.** 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol **288:**911-40.
68. **McGeoch, D. J., C. Cunningham, G. McIntyre, and A. Dolan.** 1991. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. J Gen Virol **72 (Pt 12):**3057-75.
69. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J Gen Virol **69 (Pt 7):**1531-74.

70. **McGrath, N., N. E. Anderson, M. C. Croxson, and K. F. Powell.** 1997. Herpes simplex encephalitis treated with acyclovir: diagnosis and long term outcome. J Neurol Neurosurg Psychiatry **63:**321-6.
71. **Mukerjee, R., W. Kang, V. Suri, and N. W. Fraser.** 2004. A non-consensus branch point plays an important role in determining the stability of the 2-kb LAT intron during acute and latent infections of herpes simplex virus type-1. Virology **324:**340-9.
72. **Nadelhaft, I., and A. M. Booth.** 1984. The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: a horseradish peroxidase study. J Comp Neurol **226:**238-45.
73. **Nadelhaft, I., and K. E. McKenna.** 1987. Sexual dimorphism in sympathetic preganglionic neurons of the rat hypogastric nerve. J Comp Neurol **256:**308-15.
74. **Ng, A. K., T. M. Block, B. Aiamkitsumrit, M. Wang, E. Clementi, T. T. Wu, J. M. Taylor, and Y. H. Su.** 2004. Construction of a herpes simplex virus type 1 mutant with only a three-nucleotide change in the branchpoint region of the latency-associated transcript (LAT) and the stability of its two-kilobase LAT intron. J Virol **78:**12097-106.
75. **Parr, M. B., and E. L. Parr.** 2003. Intravaginal administration of herpes simplex virus type 2 to mice leads to infection of several neural and extraneural sites. J Neurovirol **9:**594-602.
76. **Peng, W., G. Henderson, G. C. Perng, A. B. Nesburn, S. L. Wechsler, and C. Jones.** 2003. The gene that encodes the herpes simplex virus type 1 latency-associated transcript influences the accumulation of transcripts (Bcl-x(L) and Bcl-x(S)) that encode apoptotic regulatory proteins. J Virol **77:**10714-8.
77. **Peng, W., L. Jin, G. Henderson, G. C. Perng, D. J. Brick, A. B. Nesburn, S. L. Wechsler, and C. Jones.** 2004. Mapping herpes simplex virus type 1 latency-associated transcript sequences that protect from apoptosis mediated by a plasmid expressing caspase-8. J Neurovirol **10:**260-5.
78. **Perng, G. C., E. C. Dunkel, P. A. Geary, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1994. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J Virol **68:**8045-55.
79. **Perng, G. C., D. Esmaili, S. M. Slanina, A. Yukht, H. Ghiasi, N. Osorio, K. R. Mott, B. Maguen, L. Jin, A. B. Nesburn, and S. L.**

Wechsler. 2001. Three herpes simplex virus type 1 latency-associated transcript mutants with distinct and asymmetric effects on virulence in mice compared with rabbits. J Virol **75:**9018-28.

80. **Perng, G. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1996. The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript. J Virol **70:**976-84.
81. **Perng, G. C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S. M. Slanina, F. M. Hofman, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science **287:**1500-3.
82. **Perng, G. C., B. Maguen, L. Jin, K. R. Mott, J. Kurylo, L. BenMohamed, A. Yukht, N. Osorio, A. B. Nesburn, G. Henderson, M. Inman, C. Jones, and S. L. Wechsler.** 2002. A novel herpes simplex virus type 1 transcript (AL-RNA) antisense to the 5' end of the latency-associated transcript produces a protein in infected rabbits. J Virol **76:**8003-10.
83. **Perng, G. C., S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1996. A 371-nucleotide region between the herpes simplex virus type 1 (HSV-1) LAT promoter and the 2-kilobase LAT is not essential for efficient spontaneous reactivation of latent HSV-1. J Virol **70:**2014-8.
84. **Perng, G. C., S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2001. The effect of latency-associated transcript on the herpes simplex virus type 1 latency-reactivation phenotype is mouse strain-dependent. J Gen Virol **82:**1117-22.
85. **Perng, G. C., S. M. Slanina, A. Yukht, B. S. Drolet, W. Keleher, Jr., H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. A herpes simplex virus type 1 latency-associated transcript mutant with increased virulence and reduced spontaneous reactivation. J Virol **73:**920-9.
86. **Perng, G. C., S. M. Slanina, A. Yukht, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. Herpes simplex virus type 1 serum neutralizing antibody titers increase during latency in rabbits latently infected with latency-associated transcript (LAT)-positive but not LAT-negative viruses. J Virol **73:**9669-72.
87. **Perng, G. C., S. M. Slanina, A. Yukht, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2000. The latency-associated transcript gene

enhances establishment of herpes simplex virus type 1 latency in rabbits. J Virol **74:**1885-91.

88. **Sawtell, N. M.** 1998. The probability of in vivo reactivation of herpes simplex virus type 1 increases with the number of latently infected neurons in the ganglia. J Virol **72:**6888-92.
89. **Sawtell, N. M., D. K. Poon, C. S. Tansky, and R. L. Thompson.** 1998. The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. J Virol **72:**5343-50.
90. **Sawtell, N. M., and R. L. Thompson.** 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. J Virol **66:**2157-69.
91. **Sawtell, N. M., and R. L. Thompson.** 1992. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. J Virol **66:**2150-6.
92. **Sawtell, N. M., R. L. Thompson, L. R. Stanberry, and D. I. Bernstein.** 2001. Early intervention with high-dose acyclovir treatment during primary herpes simplex virus infection reduces latency and subsequent reactivation in the nervous system in vivo. J Infect Dis **184:**964-71.
93. **Smith, R. L., L. I. Pizer, E. M. Johnson, Jr., and C. L. Wilcox.** 1992. Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures. Virology **188:**311-8.
94. **Stroop, W. G., D. L. Rock, and N. W. Fraser.** 1984. Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by in situ hybridization. Lab Invest **51:**27-38.
95. **Takakuwa, H., R. B. Pyles, R. Ichimi, T. Yoshikawa, Y. Ando, A. Patel, L. R. Stanberry, and P. R. Krause.** Unpub. A mutant HSV-2 impaired for latent LAT expression is also impaired for recurrence.
96. **Tedder, D. G., R. Ashley, K. L. Tyler, and M. J. Levin.** 1994. Herpes simplex virus infection as a cause of benign recurrent lymphocytic meningitis. Ann Intern Med **121:**334-8.
97. **Thompson, R. L., and N. M. Sawtell.** 2001. Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival. J Virol **75:**6660-75.
98. **Thompson, R. L., and N. M. Sawtell.** 1997. The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J Virol **71:**5432-40.

99. **Thompson, R. L., and N. M. Sawtell.** 2000. HSV latency-associated transcript and neuronal apoptosis. Science **289:**1651.
100. **Trousdale, M. D., I. Steiner, J. G. Spivack, S. L. Deshmane, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1991. In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J Virol **65:**6989-93.
101. **Ugolini, G.** 1992. Transneuronal transfer of herpes simplex virus type 1 (HSV 1) from mixed limb nerves to the CNS. I. Sequence of transfer from sensory, motor, and sympathetic nerve fibres to the spinal cord. J Comp Neurol **326:**527-48.
102. **Vann, V. R., and S. S. Atherton.** 1991. Neural spread of herpes simplex virus after anterior chamber inoculation. Invest Ophthalmol Vis Sci **32:**2462-72.
103. **Vera, P. L., and I. Nadelhaft.** 2000. Anatomical evidence for two spinal 'afferent-interneuron-efferent' reflex pathways involved in micturition in the rat: a 'pelvic nerve' reflex pathway and a 'sacrolumbar intersegmental' reflex pathway. Brain Res **883:**107-18.
104. **Wang, K.** 2005. Personal comm., Bethesda, MD.
105. **Wang, K., P. R. Krause, and S. E. Straus.** 1995. Analysis of the promoter and cis-acting elements regulating expression of herpes simplex virus type 2 latency-associated transcripts. J Virol **69:**2873-80.
106. **Wang, K., L. Pesnicak, E. Guancial, P. R. Krause, and S. E. Straus.** 2001. The 2.2-kilobase latency-associated transcript of herpes simplex virus type 2 does not modulate viral replication, reactivation, or establishment of latency in transgenic mice. J Virol **75:**8166-72.
107. **Wang, K., L. Pesnicak, and S. E. Straus.** 1997. Mutations in the 5' end of the herpes simplex virus type 2 latency-associated transcript (LAT) promoter affect LAT expression in vivo but not the rate of spontaneous reactivation of genital herpes. J Virol **71:**7903-10.
108. **Wheatley, S. C., C. L. Dent, J. N. Wood, and D. S. Latchman.** 1992. Elevation of cyclic AMP levels in cell lines derived from latently infectable sensory neurons increases their permissivity for herpes virus infection by activating the viral immediate-early 1 gene promoter. Brain Res Mol Brain Res **12:**149-54.
109. **Whitley, R. J., and J. W. Gnann.** 2002. Viral encephalitis: familiar infections and emerging pathogens. Lancet **359:**507-13.

110. **Wilcox, C. L., and E. M. Johnson, Jr.** 1987. Nerve growth factor deprivation results in the reactivation of latent herpes simplex virus in vitro. J Virol **61:**2311-5.
111. **Wilcox, C. L., R. L. Smith, C. R. Freed, and E. M. Johnson, Jr.** 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. J Neurosci **10:**1268-75.
112. **Yang, L., C. C. Voytek, and T. P. Margolis.** 2000. Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1. J Virol **74:**209-17.
113. **Yedowitz, J. C., and J. A. Blaho.** 2005. Herpes simplex virus 2 modulates apoptosis and stimulates NF-kappaB nuclear translocation during infection in human epithelial HEp-2 cells. Virology **342:**297-310.
114. **Yoshikawa, T., J. M. Hill, L. R. Stanberry, N. Bourne, J. F. Kurawadwala, and P. R. Krause.** 1996. The characteristic site-specific reactivation phenotypes of HSV-1 and HSV-2 depend upon the latency-associated transcript region. J Exp Med **184:**659-64.
115. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res **31:**3406-15.
116. **Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel, and S. L. Wechsler.** 1990. Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. J Virol **64:**5019-28.